DETERMINATION OF QUINOLONES IN BOVINE KIDNEY USING HOLLOW-FIBER SUPPORTED LIQUID MEMBRANE EXTRACTION PRIOR TO LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

by

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DECLARATION

This dissertation is submitted in fulfillment of Master of Science degree in Analytical Chemistry to the University of South Africa. This work was carried out at Botswana National Veterinary Laboratory, Department of Veterinary Services under the Ministry of Agriculture, Botswana. I declare that this work is my own and was not submitted elsewhere before.

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SIGNATURE DATE

ABSTRACT

Focus of this study was on the development of one of the faster, simpler, cost effective and environmentally friendly sample pre-treatment techniques which employs a supported liquid membrane, in this case a Hollow-fiber supported liquid membrane (HF-SLM) for determination of seven (7) quinolone antibiotics (enrofloxacin, ciprofloxacin, danofloxacin, difloxacin, norfloxacin, nalidixic acid and sarafloxacin) in bovine kidney samples followed by LC-MS/MS analysis. The key parameters of the method were optimized and the method was validated following the 2002/657 EC guidelines. The optimum HF-SLM conditions were therefore; NaH_2PO_4 as a donor phase at pH 7, 0.1% formic acid at pH 3 as acceptor phase. Triethylamine was the optimized liquid membrane and the stirring time was optimized at 1 hour. Separation of the 7 quinolones including 3 internal standards (enrofloxacin-d_5, norfloxacin-d_5 and difloxacin-d_3) was carried out on a Phenomenex Kinetex 2.6 μm XB-C18, 100 mm x 4.6 mm, 100Å column. Validation parameters such as Correlation coefficients (r^2) ranging from 0.9714-0.9975 were obtained, while limit of detection (LOD) ranged between 3-39 ug kg^{-1} and limit of quantification (LOQ) ranged between 10-130 ug kg^{-1}. The obtained limits at which it can be concluded with an error probability of α = 95% that a sample is non-compliant (CCα) ranged from 28 – 422 ug kg^{-1} while CCβ; the smallest content of the substance that may be detected, identified or quantified in a sample with an error probability of β = 95%, ranged from 29 – 454 ug kg^{-1}. The method was found to be reproducible with CVs $\leq$ 23 %. The tested samples from Botswana local abattoirs showed no presence of quinolone antibiotics when the method was applied to real bovine kidney samples. Hollow-fiber supported liquid membrane can therefore be used for extraction of biological samples since it is a "greener technique" which uses less solvent which are less harmful to the environment when disposed as compared to dispersive Solid Phase Extraction (dSPE).

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrometry
CCα	Decision limit
CCβ	Detection capability
CE	Capillary electrophoresis
ColE	Collision energy
CXP	Cell exit potential
DEE	Diethyl ether
DLLME	Dispersive liquid-liquid microextraction
DNA	Deoxyribonucleic acid
DP	Declustering potential
Dspe	dispersive solid phase extraction
EC	European Commission
ESI	Electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GC	Gas chromatography
HF	Hollow fiber
HF-SLM	Hollow fiber-supported liquid membrane
HPLC	High performance liquid chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IS	Internal standard
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
MIC	Minimum inhibitory concentration
MMLLE	Microporous membrane liquid liquid extraction
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
MW	Molecular weight
p*K*a	Acid Dissociation constant
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RSD	Relative standard deviation
SD	Standard deviation
SDM	Single-drop microextraction

TEA	Triethylamine
US-EPA	Unites States Environmental Protection Agency
UV	Ultraviolet
Vis	Visible

EQUATIONS USED

1. LOD = $3.3*(s_y/m)$
2. LOQ = $10*(s_y/m)$
3. $S_y = \sum \sqrt{\frac{(Y_i - mx_i - b)^2}{n - 2}}$
4. LOD = Concentration of the blank + $3(SD_{blank}$/lowest standard).
5. LOQ = Concentration of the blank + $10(SD_{blank}$/lowest standard).
6. $CC\alpha = C_{MRL} + 1.64SD_{(at\ MRL)}$
7. $CC\beta = CC\alpha + 1.64SD_{(at\ CC\alpha)}$

CHAPTER 1: INTRODUCTION

1.1 Background

The food safety aspect is growing globally hence the need to monitor drugs in food from animal and animal products is necessary. Veterinary drugs are widely used in human and veterinary medicine for the treatment, control and prevention of bacterial diseases. Some of these drugs are very good for the treatment of infections in humans [1]. Examples of classes of veterinary drugs include chloramphenicol, beta-agonists, ivermectins, tetracyclines, β-lactams, aminoglycosides, macrolides, coccidiostats, sulfonamides, lincosamides and **quinolones**. Quinolone antibiotics are one of the veterinary drugs used in reared animals for treatment of diseases by farmers. They are also added to animal feeds [2] to be used as growth promoters and to increase animal mass [3-8].

Authorities in many countries worldwide are mandated with raising consumer awareness on food safety and the increasing antimicrobial resistance due to the discovery of new resistant strains of bacteria and others that are increasing over time. As a result there is increasing pressure on food safety laboratories to monitor the use of these drugs and ensure the safety of food for human consumption [9], hence the establishment of Maximum Residue Limits (MRLs) of compounds legally permitted in foodstuffs entering the human food chain as stipulated by the Food and Agriculture Organisation of the United Nations (FAO), European Union (EU), Health Canada, Japan, Food and Drug Administration (FDA) and Unites States Environmental Protection Agency (USEPA). These bodies are mandated with protecting and promoting public health and or the environment by enforcing the laws, directives and regulations related to their various objectives which include among others

production, distribution, importation, sale and/or use of consumer products, pest control products, drugs, medical devices and natural health products. The European Union's commission regulation EC No 1181/2002 [10] and commission regulation (EU) No 37/2010 [11] for example, are such instruments that have been put into place to monitor the level of these drugs in food. The directives are a guide to member countries as well as exporting countries, such as Botswana which exports beef to the EU. Council directive 96/23/EC [12] is the legislation regarding the control of antibiotic residues in live animals and animal products. Commission Decision 2002/657/EC [13] describe in detail the preferred analytical methods such as HPLC, LC-MS/MS, AAS, ICP-MS etc, and their performance criteria. According to Council directive 96/23/EC, residues are divided into two groups, which are; A and B for the purposes of monitoring and defining the legislation relating to them [14]. Group A refers to substances having an anabolic effect and are not authorized to be used while group B refers to veterinary drugs and environmental contaminants [12] such as pesticides and heavy metals. According to this classification, the groups to be monitored are as shown in Table 1.1; classification of veterinary drugs according to group A and B. Quinolones belong to group B1 of antimicrobials as shown in italics.

Table 1.1: Classification of veterinary drugs according to group A and B [14]

NAME OF GROUP		**NAME OF GROUP**	
A1	Stilbenes	B1	ANTIMICROBIALS
			Penicillins
			Quinolones
			Lincosamides
			Sulphonamides
			Tetracyclines
			Aminoglycosides
			Cephalosporins
			Macrolides
A2	Thyrostats	B2a	Anthelmentics & Avermectins
		B2b	Anticoccidials
		B2c	Carbamates & Pyrethroids
		B2d	Sedatives
		B2e	Non-Steroidal anti-inflammatory drugs
		B2f	Glucocorticoids
A3	Steroids	B3a & b	Organochlorinated Pesticides, PCBs and

NAME OF GROUP		NAME OF GROUP	
			Organophosphorus pesticides
A4	Resorcyclic acid lactones	B3c	Heavy metals
A5	Beta agonists	B3d	Mycotoxins
A6	Chloramphenicol + Nitroimidazoles + Nitrofurans		

Considerable research has been carried out on determination of quinolones in various matrices, using different extraction techniques and analysis methods. Traditional extraction techniques previously explored included liquid-liquid extraction and solid phase extraction to name a few. The recent trend is to develop miniaturized and greener sample-preparation methods which use lower quantities of solvents are environmentally friendly, faster, easy to use, inexpensive and compatible with a wide range of analytical instruments [15, 16, 17]. These techniques are termed liquid-phase microextraction (LPME), examples of which are single-drop microextraction (SDME), dispersive liquid–liquid microextraction (DLLME) and hollow-fiber supported liquid membrane (HF-SLM).

This current study explores the determination of quinolone antibiotics in bovine kidney using hollow-fiber-supported liquid membrane (HF-SLM) followed by liquid chromatography mass spectrometry. To date we are not aware of any work published before on HF-SLM in biological samples such as kidney, liver or muscle, and this has been found to be a research area of interest.

1.2 Problem statement

Since its development, work on HF-SLM by various researchers has been on determination of veterinary drugs in wastewater and biological samples such as urine and none has been done on meat samples. This research is focused on development of HF-SLM as a sample preparation and/or clean-up for biological samples such as meat, kidney and liver. In addition challenges involved when using HF-SLM in kidney samples were considered.

1.3 Rationale

The importance of this study is to move away from conventional extraction methods specifically solid phase extraction and liquid-liquid extraction which uses large amounts of solvents posing a challenge to the environment due to the difficulty and expenses related to disposal. The study proposes a greener technique such as hollow fiber-supported liquid microextraction which affords comparable extraction efficiencies and clean samples, faster and also in an environmentally friendly way.

1.4 Aims and objectives

The aim of this study is to develop a greener sample preparation method for detecting and quantifying quinolone antibiotics in bovine kidney samples using HF-SLM followed by LC-MS/MS analysis. This was achieved through the following specific objectives;

- To develop an LC-ESI-MS/MS separation method for quinolones in bovine kidney samples,

- To validate the separation method based on the following validation parameters: (a) Linearity; (b) Precision; (c) Limit of detection (LOD) and (d) Limit of quantification (LOQ).
- To develop and optimise two sample extraction methods (i) hollow fiber – supported liquid membrane (HF-SLM) and (ii) dispersive solid phase extraction (dSPE).
- To validate HF-SLM and dSPE methods according to Commission Decision 2002/657 EC.
- To compare the performance merits between HF-SLM and dSPE.
- To apply the developed HF-SLM method to real bovine kidney samples.

1.5 Dissertation Overview

This dissertation is made of five chapters; **Chapter 1** introduces the work and the objectives to be attained from the research work while **Chapter 2** covers the literature review of the main research topic of quinolones and their extraction from biological samples using a "greener technique". It also covers comparison of different extraction techniques and the analytical methods of analysis. The Experimental procedures used in the study are covered in **Chapter 3.**

Chapter 4 shows the results obtained from the experiments conducted when extracting samples using the Hollow fiber- supported liquid membrane compared to those obtained through dispersive solid phase extraction. **Chapter 5** presents conclusions of the research undertaken and recommendations for future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Quinolones are a family of synthetic broad-spectrum antibiotics [18]. Nalidixic acid was the first quinolone antibiotic to be discovered in the early 1960s. Development of other compounds such as flumequine, norfloxacin and enoxacin was seen after a decade and became available for clinical use [19]. The structures of these molecules are based or derived from the heterobicyclic aromatic compound called quinoline (Figure 2.1), the name of which originated from the oily substance obtained after the alkaline distillation of quinine [19, 20]. A wide variety of quinolone compounds are a product of animals, plants and microorganisms reported to have been discovered from the formation of quinine compound [21]. The basic structure of the fluoroquinolone class is therefore based upon the quinoline ring system [22, 23]. Many researchers have developed wide interest (scientifically and clinically) around these analytes since their discovery [22]. In addition to their uses in animal husbandry, they are also used in human pharmaceuticals for treatment of urinary tract infections [19, 24].

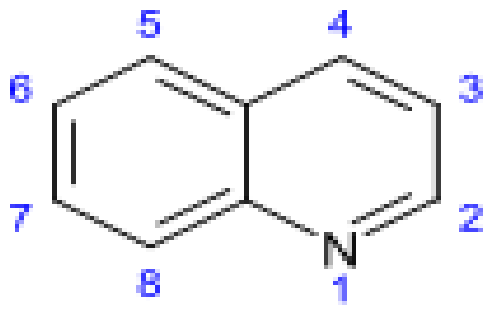


Figure 2.1: Structure of quinoline, shown with its accepted numbering scheme to indicate sites of substitution [22, 23].

2.2 Chemistry of quinolones

Most quinolones belong to the subset fluoroquinolones, due to the fluorine atom attached to the central ring system, normally attached at Carbon 6 or 7 positions as shown in Figure 2.2. The blue substituted R in most cases is a piperazine; the compound becomes a fluoroquinolone if the connection shown in red is fluorine. This basic fluoroquinolone molecule was also studied by Andersson and MacGowan [19, 22, 25]. They stated that "one of the earliest changes to the structure was addition of a fluorine molecule at position 6" [19, 26]. The single alteration provides a more than 10-fold increase in gyrase inhibition and up to 100-fold improvement in minimum inhibitory concentration (MIC) [27]. This inhibition was also studied by Sarkozy [28]. The development of the two major groups of quinolones and naphthyridones were reported by Domagala (1994). They discovered that the presence of nitrogen at position 8 identifies another group termed naphthyridones, example of which is nalidixic acid. A carbon and associated group at position 8 identifies the quinolones, as reported in a paper by Ball (2000) [19, 29]. The structures of these two groups (quinolones and naphthyridones) were reported to have been further enhanced by the addition of groups to the N1, C-5 and C-7 positions of their respective basic molecules. The discovery continues to suggest that "the addition of piperazine to the C-7 position has effectively improved activity against Gram-negative organisms. This is well illustrated in the structure for norfloxacin". New research suggests that a piperazine ring may play a role in inhibiting efflux mechanisms, thereby improving the potency of these drugs. The structure of norfloxacin is a good example to illustrate these developments and is extended to all quinolones (except

garenoxacin) which has fluorine at position 6 while other analytes have six-membered rings at position C-7 [19].

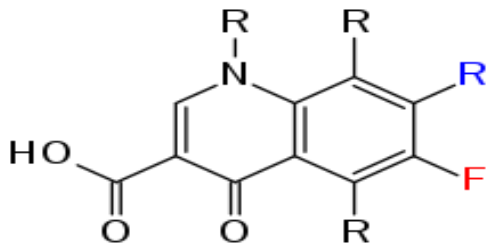


Figure 2.2: Basic structure of quinolones

2.3 Generations of Quinolones

Quinolones are divided into generations according to their antibacterial performance [29]. There are four generations though the first generation is rarely used nowadays. Some quinolones from the second, third, and fourth generation drugs are reported to have been removed from clinical practice due to severe toxicity issues or discontinued by their manufacturers. The generations are as follows;

1. First generation agents; these are compounds reported to have had false antimicrobial activity as a result of the addition of a piperazine substituent at position 7 of the naphthyridone core [29].
2. Second generation agents have replaced the first generation and predominantly have Gram-negative activity. The next significant step was seen with formation of generation IIb, with improved Gram-positive activity [30]. Examples of the first and second generations agents are as summarized in the table below.

Table 2.1: First and second generations agents

FIRST GENERATION	**SECOND GENERATION**
Cinoxacin	Ciprofloxacin
Flumequine	Enrofloxacin [31]
Nalidixic acid	Sarafloxacin [31]
Oxolinic acid	Difloxacin [31]
Pipemidic acid	Enoxacin
	Norfloxacin
	Lomefloxacin

3. Temafloxacin and sparfloxacin forms the third generation agents which are characteristic of having enhanced activity against Gram-positive pathogens [29].
4. The last generation, which is called fourth generation agents, is made of clinafloxacin and gatifloxacin.

Quinolone antibacterial agents are compounds which pose both negative and positive moieties and are termed amphoteric and are considered zwitterionic. Their pK_a is between acidic and basic due to the carboxylic acid and one or more amine (which are basic) functional groups attached to the aromatic structures [28, 32]. The behaviour of quinolones is compared to 1-naphthoic acid due to their structural similarity. 1-naphthoic acid is reported to have one pK_a of 3.69 which corresponds to the carboxylic group attached to the benzene ring of the quinolones structure. Due to these reason, p$K_{a,\,1}$ of flouroquinolones was assigned to the carboxylic acid [33]. The remaining pK_a's values were assigned

to the three nitrogen sites based on their electron density which increases from moieties B, C to D. Therefore p$K_{a,2}$ was assigned to moiety B, p$K_{a,3}$ to C and p$K_{a,4}$ to D as reported by Buckingham et al. [34]. Figure 2.3 illustrates the p*K*a's corresponding to the moieties. Table 2.2 shows the physicochemical properties of the nine quinolones including three internal standards studied in this work.

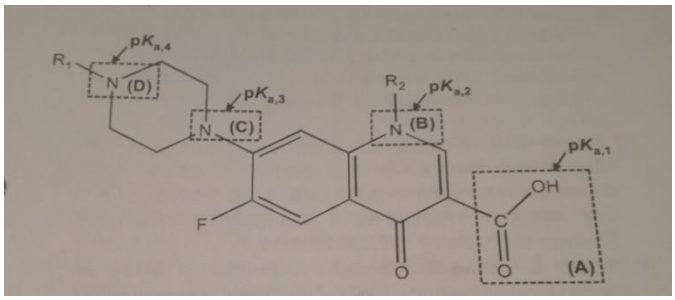


Figure 2.3: Moieties corresponding to the p*K*a values [34].

2.4 Uses of quinolones

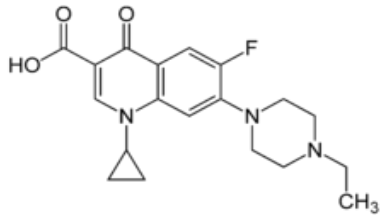
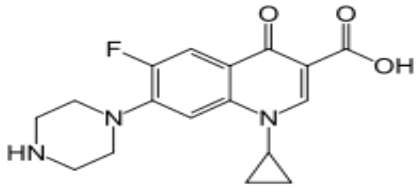
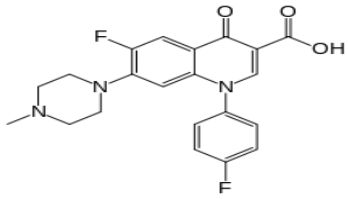
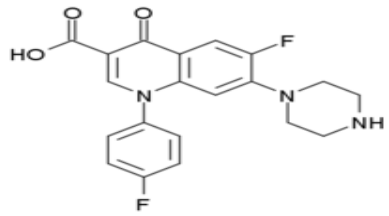
Studies have shown that quinolones act by not allowing for duplication and unwinding of bacterial DNA [35]. Ronald and Low (2003) also made a discovery which stated that "quinolones in food of animal origin lead to the development of resistance against the quinolone bacteria. These bacteria are transmitted to humans through direct contact with the animal or when consuming food and water contaminated with these drugs. An example of resistance to quinolones which is reported to have been observed since the early 1990s is the transmission of the most common and dangerous bacteria called campylobacter which is transmitted from animal-to-human. This was also reported in a paper by Zhao and Stevens, Agilent applications note [18, 36].

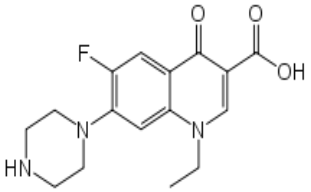
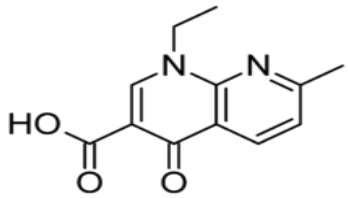
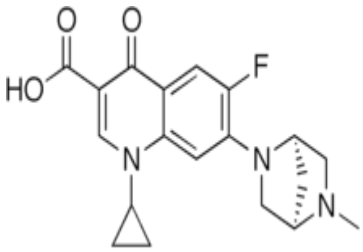
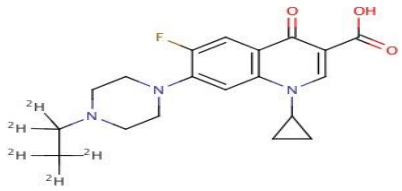
Use of quinolones in livestock has been identified as a particular area of concern because of their significance in treatment of a broad range of infections in humans including gastrointestinal infections caused by zoonotic bacteria transmitted to humans via the food chain. Nalidixic acid was the first quinolone used clinically in animals. Subsequent quinolones, all congeners of nalidixic acid synthesised in the late 1960s and 1970s, clearly showed both improved antibacterial and pharmacokinetic properties as well as reduced side effects. Some of these are, for example, flumequine and oxolinic acid which are still used in veterinary medicine in a limited number of countries. Clinically, a significant breakthrough was achieved by the introduction of the fluoroquinolones which are available for therapy of animals in worldwide. However, the usage of these fluoroquinolones differs greatly from species-to-species, their occurrence and locations [3].

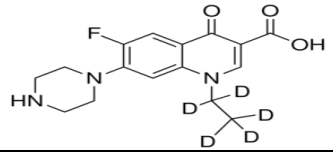
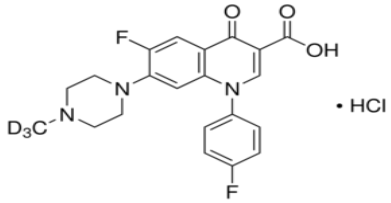
2.5 Limitations and/or challenges

Abuse and misuse of veterinary drugs and non-compliance to withdrawal periods during administration to animals can leave some residues of these drugs in edible animal tissues, affecting people's health. These can lead to a health scare due to possible toxic effects, development of resistance to medicines, allergies, etc. as well as environmental and industrial problems [3-6, 37-40]. The health problems of toxic effects and quinolone-resistance mentioned above are the effects of these drug residues and/or their metabolites in meat and meat products [41]. Abuse and misuse of veterinary drugs greatly affects international trade of food products [42]. The effect on the country's trade market due to their food products testing positive to some of these drugs could be irreparable especially to emerging economies. Monitoring of these antibiotics in food is therefore very vital.

Table 2.2: Physicochemical properties and structures of quinolones

Compound name/structure	**MW (g mol^{-1})**	**CAS No.**	**p*Ka* [43]**	**MRL (µg kg^{-1}) in bovine kidney [11]**
Enrofloxacin 	359.4	93106-06-6	3.85±0.30; 6.19±0.18	200
Ciprofloxacin 	331. 3	85721-33-1	3.01±0.30; 6.14±0.13; 8.70±0.09; 10.58±0.30	200
Difloxacin 	399.4	98106-17-3	5.66±0.04; 7.24±0.06	800
Sarafloxacin 	385.4	98105-99-8	6.00; 8.6	200

Compound name/structure	**MW (g mol^{-1})**	**CAS No.**	**p*Ka* [43]**	**MRL (µg kg^{-1}) in bovine kidney [11]**
Norfloxacin 	319.3	70458-96-7	6.22, 8.38	100
Nalidixic acid 	232.2	389-08-2	5.95	100
Danofloxacin 	357.4	112398-08-0	6.07±0.06, 8.56±0.07	400
Enrofloxacin-d_5 	364.4	1173021-92-5		Internal standard (IS)

Compound name/structure	**MW (g mol^{-1})**	**CAS No.**	**p*Ka* [43]**	**MRL (µg kg^{-1}) in bovine kidney [11]**
Norfloxacin-d_5 	324	1015856-57-1		Internal standard (IS)
Difloxacin-d_3 	403	1173021-89-0		Internal standard (IS)

2.6 Sample extraction methods for quinolones

Sample preparation is a very important part of analytical procedures and it has been reported to be time consuming and not cost effective [44]. It is a pre-requisite to any analytical method since it has been discovered that analysis of pure samples which has not been extracted or treated can harm the equipment [15]. Sample preparation involves pre-treatment which includes isolation, clean-up and/or pre-concentration of the analyte. The basic concept of sample-preparation methods is to convert a real matrix into a sample suitable for analysis [45]. Various traditional sample preparation methods such as liquid liquid extraction (LLE), solid phase extraction (SPE), Soxhlet extraction [46], automated microdialysis [47] and automated extraction by turbulent flow chromatography [48] have been applied

before for determination of quinolones. SPE has been applied by various authors for analysis of quinolones in various matrices [16, 39, 49-54]. Liquid-liquid extraction has also been applied for extraction of quinolones from poultry muscle [52, 54]. The conventional sample-preparation techniques (LLE, SPE, Soxhlet, etc.) mentioned above have been found to have drawbacks such as complications during disposal, time-consuming and labour intensive; requirement of large amounts of organic solvents which are toxic to the environment during disposal and carcinogenic.

Usage of large volumes of solvents which are harmful to the environmental as well as expensive to dispose are slowly being minimized and replaced with environmental friendly solvents where possible [15]. This has led to a pressure to develop sample preparation methods which are fast, precise, accurate and sensitive [15]. As presently discovered, the main aim is to change to simplified and miniaturized sample preparation techniques and decreasing the quantities of environmentally unfriendly organic solvents used [15, 16, 17]. The principles of green chemistry, introduced by Anastas and Warner are directly related to analytical chemistry. They stated that "the most important green chemistry principle is; to prevent generation of waste, use of safer solvents and auxiliaries and design for energy efficiency" [55]. Simple, inexpensive sample preparation methods that use minimal solvents such as liquid-phase microextraction (LPME) have been introduced recently. LPME is a technique which uses less amount of solvent for sample pre-treatment as compared to LLE. Small microliter volumes of solvent are required to concentrate analytes from various samples rather than hundreds of millilitres needed in traditional LLE. It is compatible with gas chromatography (GC), capillary electrophoresis (CE) and HPLC.

In LPME, extraction takes place from an aqueous sample containing analytes termed THE donor phase into a small amount of a water-immiscible solvent termed THE acceptor phase. The three main categories of LPME are: (i) single-drop microextraction (SDME), (ii) dispersive liquid–liquid microextraction (DLLME) and (iii) hollow-fiber microextraction (HF-LPME) [15]. HF-LPME was introduced by Pedersen-Bjergaard and Rasmussen [56] using the basic principle of supported liquid membrane (SLM). The supported liquid membrane was developed by Audunsson as a sample clean-up and/or pre-concentration of amines in a flow system [57]. The principle of SLM was developed by the Lund research group in Sweden following the publication by Audunsson. Various authors have applied supported liquid membrane as published in various research papers [58-62]. A membrane is defined as a semi-permeable barrier between two phases. Considering membranes as phase separators, this concept can be extended to liquids as well. A liquid that is immiscible with the donor phase and receiving phase solutions and serves as a semi-permeable barrier between these two liquid phases is called a liquid membrane [63, 64]. Liquid membrane based extraction is a combination of liquid-liquid extraction and membrane separation in a single device.

Liquid membrane extraction is divided into three-phase systems and two phase systems. Supported liquid membrane (SLM), is a membrane extraction based on a three-phase system whereby a thin film of an organic phase is immobilised in a hydrophobic porous polymer membrane, which is placed between two aqueous liquids (the donor and the acceptor solutions) [45]. This technique allows for the extraction and pre-concentration of ionisable analytes by an appropriate pH adjustment of both the sample (donor solution) and the extractant phase (acceptor solution) [16]. Phosphate buffers or alkalis are more preferred for extraction of analytes from sample matrix instead of organic solvents [23]. The

pH of the buffer containing the extracted sample is adjusted in such a way that the analytes are in their neutral form in the donor solution [16]. This principle is suitable for the extraction of polar compounds which are acidic or basic, charged, metals and it is compatible with reversed phase HPLC [65]. To extract basic compounds, it is important to adjust the pH of the sample to alkalinity such that solubility of the analytes is suppressed while pH in the acceptor solution should be low in order to promote analyte solubility. This set-up allows for easy extraction of basic compounds from the donor solution through the membrane solvent and further into the acceptor phase without being extracted back to the membrane solvent again [15]. By contrast, for acidic analytes pH in the sample should be low and an alkaline acceptor solution should be added inside the lumen of the fiber to enhance extraction in the same manner as for basic compounds [15].

The two-phase system (aq/org), also called microporous membrane-liquid liquid extraction (MMLLE) is a technique where analytes are extracted into an organic solvent separated from the aqueous sample by a hydrophobic porous membrane. This approach is suitable for more hydrophobic analytes and is compatible with gas chromatography [66]. Msagati and co-workers studied sample preparation using supported liquid membrane extraction techniques [67]. Msagati and Nindi applied supported liquid membrane as a sample preparation method for various veterinary drugs such as benzimidazole anthelmentics, sulphonamides, macrolides, aminoglycosides, anabolic steroids and stilbenes in various matrices [68-74].

The principle of HF-LPME and HF-SLM has been reported to be similar. A measured piece of a porous hollow fiber (HF) used for extraction can be a rod configuration with one end closed at the

bottom (Figure 2.4a). Other possible configurations observed are the u-shape or loop configuration where both ends are connected to guiding tubes (Figure 2.4b). Alternatively, the hollow fiber can be attached directly to the needle of a microsyringe for easy injection and withdrawal of the acceptor solution, as illustrated in Figure 2.4c [75]. These fibers are then immersed in a sample bottle containing an aqueous sample solution.

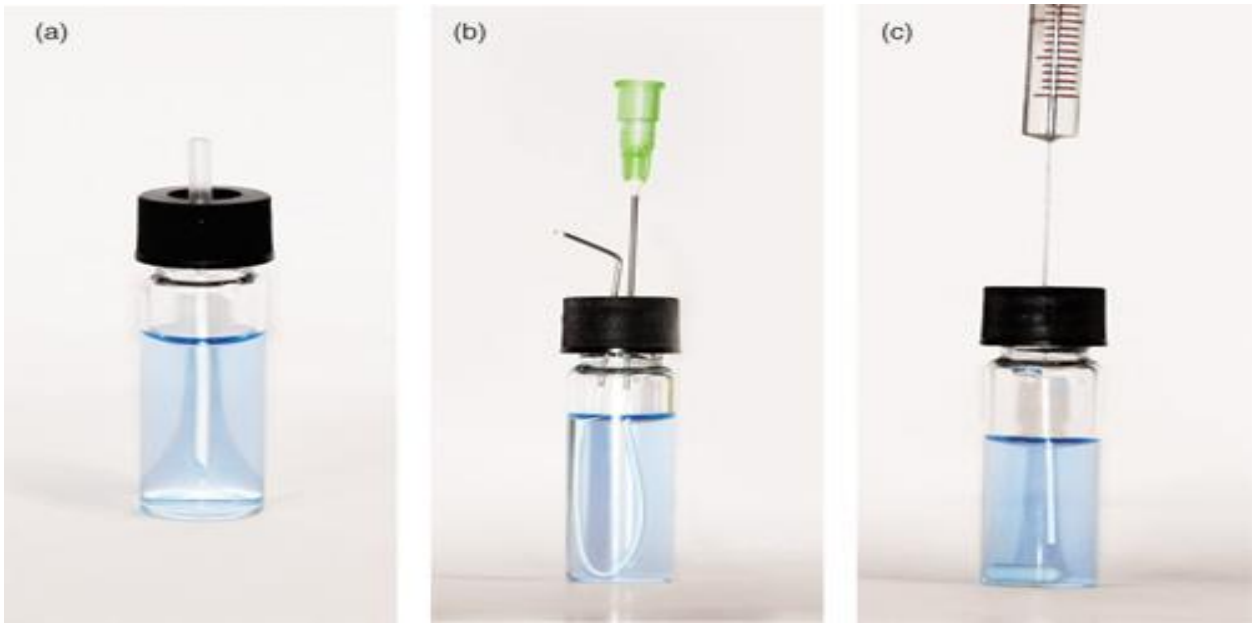


Figure 2.4: Connection configurations; a) rod configuration, b) u-shaped or loop configuration, c) hollow fiber attached directly to the needle of a microsyringe [75].

To prepare the fibers for extraction, they are first dipped in the organic solvent which acts as a supported liquid membrane, for a few minutes to immobilize solvent in the pores, and excess solvent washed off with water [76]. This is followed by filling the fiber with acceptor phase using a syringe. The organic (membrane) solvent plays a dominant role in three phase LPME affecting both recoveries and extraction kinetics [77]. The main property of the solvent used as a membrane solvent is that it

should be immiscible with water and less polar to ensure that it remains within the pores of the fiber during the extraction without leaking into the aqueous sample. It should also be of low volatility to prevent it from evaporating and causing leakage as well; lastly it should be of low viscosity to ensure increased flow of analytes in the same direction, termed "rapid mass transfer" [77]. Having met all the properties mentioned, the organic solvent is expected to form a thin layer within the wall of the HF. It is also very important that the extraction solvent also called acceptor solution be compatible with the HF to ensure complete filling of the walls of the HF. The acceptor solution is then injected inside the lumen of the HF. The organic solvent that has been used as the membrane solvent can be used as an acceptor solution as well; in that case the extraction is called a two-phase system. The acceptor solution may be an acidic or alkaline aqueous solution depending on the polarity of analytes being extracted, resulting in a three-phase extraction system [76].

To describe extraction in the two-phase LPME system, the target analytes are extracted from the aqueous sample and into the organic solvent (acceptor solution) present both in the porous wall and inside the lumen of the HF [15, 78]. Solvents which can be used as supported liquid membranes are normally the long chain hydrocarbons like n-undecane or kerosene and more polar compounds like di-hexyl ether, di-octyl phosphate, [66] triethylamine and diethyl ether.

Hollow fibers are made of polypropylene and are manufactured in different sizes with most common diameters of 600 μm or 300 μm. All different kinds of hollow fibers reported by different authors are based on the Accurel®PP fiber materials produced by the company Membrana GmbH (Wuppertal, Germany) [66, 79-81].

The major advantages of HF-LPME can be summarized as follows:

- High enrichment (up to 25,000-fold) [77]
- Excellent sample clean-up
- Direct compatibility with HPLC, CE and MS
- Low solvent consumption (10–30 μL of solvent for each extraction) [75].

In terms of applications of greener techniques in the determination of quinolones in meat samples, Moema et al. applied DLLME, for determination of flouroquinolones in biological matrices (chicken liver) [42]. Wang et al. recently published work on the determination of four flouroquinolones in meat also by DLLME [82]. Thus far HF-SLM in quinolones has been applied in surface water [79], wastewaters [83] and biological matrices such as urine only [7]. Dispersive Liquid –Liquid Microextraction (DLLME) is one of the "greener" sample preparation techniques which were also developed to overcome the limitations of liquid phase extraction and clean-up posed by the traditional methods. It is a ternary solvent, miniaturized liquid-liquid extraction method which uses microliter volumes of extraction solvent based on the equilibrium distribution process of the target analytes between sample solution and extraction solvent [84]. DLLME is similar to HF-SLM in that they provide high enrichment, use less solvent and are not time consuming. On the other hand, DLLME is advantageous over HF-SLM due to its high efficiency in terms of sensitivity and recovery [85]. The main challenges that both methods presents, is extraction of analytes from biological samples which needs to be extracted first from the solid matrix into an aqueous phase prior to DLLME or HF-SLM procedure, as compared to water samples where the anaytes are in aqueous phase.

2.7 Analysis of quinolones

It is very important nowadays to use sensitive and fast methods which can perform multiple-residue screening for determination of veterinary drugs. For quinolones, various methods using liquid chromatography with UV or fluorescence detection have been developed for applications in various matrices and species such as bovine, porcine, poultry, fish, feed and wastewater effluents [52, 53, 86, 87]. High performance liquid chromatography (HPLC) coupled with diode array detection (DAD) was also used for analysis of quinolones extracted from water by supported liquid membrane single hollow fibers [79]. DADs identify substances according to their retention time and their quantification is based on peak intensity and peak area [88, 89]. This detector is limited since it is not able to differentiate between compounds which are structurally similar (i.e isomers), or those that elute at the same time especially during simultaneous multiple-residue analysis making it difficult to accurately identify and quantitate compounds. HPLC techniques are also limited in terms of sensitivity and sample throughput.

To improve on sensitivity and specificity, mass spectrometry (MS) became more preferred. The principle of this technique is based on separation of ions in vacuum based on their mass-to-charge (m/z) ratios and measures the intensity of each ion. It is a highly sensitive detector which provides qualitative and quantitative information concerning molecular weights and structure of compounds. When coupled to the LC system, parameters such as resolution in separation and quantitative capabilities are improved [88].

Liquid chromatography tandem mass spectrometers (LC-MS) utilizing to electrospray ionisation are increasingly being used and have become the preferred system for separating and identification of complex mixtures. This kind of system is built in such a way that it can perform two or more mass spectrometry experiments, termed MS^n which refers to its ability to perform multiple ion production and filtering within a single instrument [90]. Their usages are mostly in the fields of pharmaceuticals, environment, foods, and industrial materials. The technique combines high specificity, sensitivity and allows for rapid and multiple-residue determination in complex matrices, together with structural information. It is also able to perform multiple reaction monitoring (MRM), which allows for identification and quantification of multiple analytes in a single run, without the need to resolve them. These can be seen from the extensive research work done on analysis of quinolones in various matrices using LC-ESI-MS/MS [18, 91-97]. The sensitivity obtained for eight quinolones studied in the paper by Lombardo-Agui et al. ranged between 8.0×10^3 to 2.5×10^4 [97], while sensitivity obtained for four quinolones by Martos et al. ranged between 5.4×10^3 and 2.7×10^4 [92]. The extraction efficiencies obtained for four quinolones extracted from beef kidney using solid phase extraction and later analysed with HPLC electrospray mass spectrometry ranged from 64.1 to 143.0 % [91]. In another paper in which a group of veterinary drugs including four quinolones were extracted using liquid liquid extraction and analysed by LC-MS/MS, extraction efficiencies ranged between 60 – 80 % [94] and 67.0 – 95.8 % [96].

The 4000 Q-trap system used in this study provides high specificity, selectivity, ability to perform multiple reaction monitoring (MRM) with a high level of sensitivity and is able to quantify both small

molecules and peptides. As the name suggests the system is equipped with a quadrupole mass analyser. It is connected to an LC system and utilizes electrospray ionisation.

CHAPTER 3: EXPERIMENTAL

3.1 Materials and Reagents

Enrofloxacin (ENRO) (99.0%), ciprofloxacin (CIPRO) (99.5%), norfloxacin (NORFLO) (99.8%), sarafloxacin hydrochloride trihydate (SARA) (97.3%), enrofloxacin-d5 hydrochloride (ENRO-d5) (99.3%) (IS), difloxacin-d3 hydrochloride (DIFLO-d3) (99.4%) (IS), norfloxacin-d5 (NORFLO-d5) (99.4%) (IS), difloxacin (DIFLO) (99.4%), danofloxacin (DANO) (99.8%), formic acid, methanol (LC-MS Chromasolv), phosphoric acid (85%), hydrochloric acid (37 %) were all from Fluka (Steinheim, Germany). Nalidixic acid (NALI) (≥98%), acetone (LC-MS Chromasolv), triethylamine, sodium dihydrogen phosphate (NaH_2PO_4) and acetonitrile (LC-MS Chromasolv) were from Sigma-Aldrich (Steinheim, Germany). Sepra C18–E 50 µm, 65 Å bulk packing sorbent was from Phenomenex (Torrance, CA, USA). The membrane filters, 0.45 µm Polyvinylidene Fluoride (PVDF) were from Acrodisc® Syringe Filters (Pall Corporation, New York, USA).

Water was purified using a Waters Milli-Q purification system (Millipore, Billerica, MA, USA) at a resistivity of 18.2 MΩ cm^{-1}. The hollow-fibers made of Accurel®PP fiber, diameter of 600 µm were a gift from Professor Jan Ake Johnson, of the Chemistry Department, Lund University, Sweden. Nitrogen gas (N_2) of 99.9 % purity used for drying samples was supplied by Air Liquide (Johannesburg, South Africa).

3.2 Instrumentation

3.2.1 Mass Spectrometer

An Applied Biosystems 4000 Qtrap mass spectrometer (Figure 3.1) was from Applied Bio systems /ABSciex (Pty) LTD (Darmstadt, Germany) and was used for all mass spectral measurements. The mass spectrometer was equipped with a TurboV source using electrospray ionization (ESI) in positive ion mode. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode to confirm the identity of quinolones. This was achieved by selecting specific precursor-to-product ion for each quinolone and quantifying using the most abundant transition.

Figure 3.1: AB Sciex 4000 Qtrap MS/MS

3.2.1.1 Optimization of MS/MS parameters

The MS/MS parameters were generated by first infusing the standard solutions directly in the mass spectrometer in order to optimize the ion source parameters. The individual analytes at concentrations of 10 ng μL^{-1} were mixed with methanol/0.1 mM ammonium formate in UHP water (50/50, v/v) using a 1.0 mL Hamilton syringe and pushed through a Harvard apparatus model 11 syringe pump into the mass spectrometer at a flow rate of 10 µL min^{-1} until parameters were established. The purpose was to determine the precursor ions, product ions, declustering potential (DP)/cone voltage, collision energies (CE), cell exit potential (CXP) and dwell time. Other parameters including ion source voltage, temperature (550℃), ion source gas 1 (40 psi) and 2 (40 psi), curtain gas (30 psi) and entrance potential (10 V) were optimized on an AB Sciex API 4000 QTRAP ESI-MS/MS.

3.2.2 HPLC

Chromatographic analysis was carried out on an Agilent 1200 series HPLC equipped with a binary pump, an on-line degasser, an autosampler, automatic injector and a thermostated column compartment. The system was operated with Analyst 1.5.2 software. In preliminary studies the separation method was developed using a Phenomenex Luna 3µm C18 150 mm x 2.00 mm, 100 Å column and a gradient method consisting of mobile phase A (40 mM ammonium formate pH 2.8) and B (methanol + 0.1% formic acid) at a flow rate of 0.3 mL min^{-1}, column temperature of 23℃ and injection volume of 20 µL. Finally, LC separation of quinolones was achieved on a Phenomenex Kinetex 2.6 µm XB-C18, 100 mm x 4.6 mm, 100 Å column (Torrance, CA, USA) using a gradient elution method (Table 3.1).

Table 3.1: Gradient profile - Mobile phase A: 0.1% formic acid in water; B: methanol; Flow rate 400 µL min^{-1}, injection volume 20 µL; Column temperature: 40℃

Step No	Time (min)	A (%)	B (%)
1	0.00	70	30
2	1.00	70	30
3	7.00	15	85
4	9.00	15	85
5	9.10	70	30
6	13.10	70	30

3.2.3 Other instrumentation

A food chopper (Robot Coupe R201 Ultra E, France) was used for homogenising samples and an analytical balance from Radwag AS 220/C/2 (Poland) was used for weighing standards. A technical balance from Adam PGW (Danbury, USA) was used for weighing samples, SPE bulk sorbent and NaH_2PO_4. A centrifuge, Heraus Biofuge Primo R (Thermo Electron Corporation, Germany) capable of 4000 rpm and adjustable temperature between 4 - 10 °C was used for separation of the liquid from the solid particles. The pH of samples and NaH_2PO_4 were adjusted using a Seven multi pH meter (Mettler Toledo, Switzerland). The pH meter was first verified using the pH 4.0, 7.0 and 10.0 buffers. Verification passed when the obtained slope was 95%. Evaporation of samples was done using a sample concentrator (Turbo-vapLV, Caliper Life Sciences, Charlotte, USA), and samples were mixed using a vortex mixer (Multireax, Heidolph Instruments, Germany) and Biomega magnetic hotplate stirrer (Edison NJ, USA) for stirring samples.

3.3 Analytical procedures

3.3.1 Preparation of stock standard solutions

Stock standard solutions, at a concentration of 1 mg mL^{-1} were prepared by accurately weighing an amount corresponding to 10 mg after correcting for purity, water of hydration and salts added to standards for each standard material (CIPRO, SARA, DANO, NORFLO, NALI) and NORFLO-d5 (IS) (0.5 mg mL^{-1}) into a weighing boat, dissolving and making up to 10 mL with acetonitrile/0.1% formic acid in water (50:50, v/v). The same amounts of ENRO, DIFLO, DIFLO-d3 (IS) and ENRO-d5 (IS) were dissolved and made up to volume with methanol/0.1% formic acid in water (50:50, v/v). The standards were stored at -20 °C until the time of analysis.

3.3.2 Preparation of spiking solution and internal standards mixtures

A spiking solution mixture containing all the seven analytes was prepared in a 100 mL volumetric flask by pipetting different volumes of the individual stock standard solution basing on the MRLs or action levels of each analyte, to prepare the desired concentration. A separate mixture of internal standards was also prepared in a 100 mL volumetric flask. Both solutions were prepared in methanol and stored at -20℃. The concentrations of the spiking solution and internal standards mixtures are as tabulated in Table 3.2.

Table 3.2: Concentrations of the spiking solution and internal standards mixtures

Analyte (MRL/ Action level)	Concentration of stock solution (µg mL^{-1})	Aliquot from stock solution (µL)	Final volume (mL)	Final concentration of analyte (µg mL^{-1})
Cipro (100)	1000	100	100	1
Enro (100)	1000	100	100	1
Dano (200)	1000	200	100	2
Diflo (400)	1000	400	100	4
Norflo (50)	1000	50	100	0.5
Nalidixic (25)	1000	25	100	0.25
Sara (100)	1000	100	100	1
Enrofloxacin - d5 (IS)	1000	500	100	5
Difloxacin – d3 (IS)	1000	500	100	5
Norfloxacin – d5 (IS)	1000	500	100	5

3.3.3 Preparation of 0.1 % phosphoric acid

Six hundred (600) µL of phosphoric acid (85 %) was pipetted into a 500 mL volumetric flask containing half- filled deionised water, and made to the mark with deionised water. This reagent was mixed with acetonitrile and used for extraction of the kidney samples prior to hollow-fiber procedure.

3.3.4 Preparation of 0.1 % formic acid

Five hundred (500) µL of concentrated formic acid was pipetted into a 500 mL volumetric flask containing deionised water or acetonitrile. When mixed with acetonitrile, this reagent was used during preparation of stock standard solutions, and when in aqueous solution it was used as a mobile phase.

3.3.5 Preparation of sodium dihydrogen phosphate buffer (20 mM) (NaH_2PO_4)

The reagent was prepared by weighing 1.2 g of sodium dihydrogen phosphate in a beaker then dissolving with deionised water. The solution is then transferred quantitatively into a 500 mL volumetric flask and filled to the mark with deionised water. The pH was checked and adjusted to 7.0 with sodium hydroxide solution

3.3.6 Sampling and sample preparation

The bovine kidney samples were sampled from Botswana National abattoirs based on Council Directive 96/23/EC [12] or superseding legislation. The directive describes that **s**amples shall be properly sampled, handled properly which involves packaging and transportation in appropriate conditions and processed which involves proper storage conditions and sample preparation procedures leading to having a high chance of detecting the substance in the sample. Sample handling procedures that will prevent the possibility of accidental contamination or loss of analytes are supposed to be created and implemented [12]. The samples were stored at -20 °C until analysis. Blank samples were obtained by first screening collected bovine kidney samples using LC-ESI+MS/MS to ensure that they

were free of the analytes of interest. About 10 g of various kidney samples were homogenized using a mini food chopper and stored at -20 °C prior to extraction. Two grams ± 0.02 g of blank tissue samples were weighed into 50 mL polypropylene centrifuge tubes. These samples were used for the matrix-matched calibration curve and some for the recovery studies. For recovery studies, samples were spiked at ½, 1 and 1.5 MRL corresponding to 100 µL, 200 µL and 300 µL of the spiking solution prepared in 3.3.2, while the matrix-matched calibration samples for the calibration curve were spiked with 0, 100, 200, 300, 400 and 500 µL of the spiking solution. The internal standard mixture (200 µL) also prepared in 3.3.2; was added to all samples including calibration standards. Samples were vortex mixed to enhance interaction of analytes with the matrix.

3.3.7 Developing a hollow-fiber-supported liquid membrane (HF-SLM) method

Five milliliters of a mixture of 0.1% phosphoric acid: acetonitrile (30:70 v/v) followed by 10 mL of NaH_2PO_4 (pH 7) were added to the weighed and fortified 2 g kidney samples. The samples were mechanically shaken with a multi reaction mixer for 10 minutes then centrifuged at 4000 rpm for 10 minutes. The extracted supernatants were transferred into clean tubes and their pH checked to ensure that they were neutral. Hollow fibers were prepared by cutting about 4 cm length strips of the hollow fibers (HF), washing with acetone, drying and sealing one end using a heated spatula. The HFs were impregnated with the membrane by dipping in a liquid membrane (triethylamine) for a few seconds then filled with ~ 20 µL of the acceptor solution (0.1 % formic acid) using a syringe. Excess of the liquid membrane was then washed off with water. The HF, supported by a needle, was immersed in the bottle containing the sample solution (donor phase). The samples were stirred using a magnetic stirrer at 200 rpm for 1 hour. Using a syringe, the samples (10 µL) were pushed through the HF into

a glass insert and analysed. In order to obtain good results in HF-SLM, key parameters have to be optimised as described in the next section.

3.3.8 Optimisation of hollow-fiber supported liquid membrane

It is important to optimize parameters that affect quality of results of HF-SLM. The optimized parameters are as described below.

3.3.8.1 Selection of liquid membrane

Triethylamine (TEA) and diethyl ether (DEE) were investigated as possible liquid membranes. The experiments were performed in such a way that some parameters were held constant while varying the liquid membranes. Formic acid (0.1%) was used as the acceptor phase, while NaH_2PO_4 was used as a donor phase. Extraction or stirring was done for 1 hour. Six bovine kidney samples (2 g) were weighed, fortified with spiking solution containing seven analytes as well as internal standards mixture and subjected to the HF-SLM procedure as described in 3.3.7 above. Half of the samples were subjected to triethylamine as a liquid membrane while the other half was used with diethyl ether.

3.3.8.2 Optimisation of pH of donor phase

It is very necessary to adjust pH of the sample in LPME in so as to increase the diffusion rate of analytes passing through the liquid membrane into the acceptor phase [77]. To select an optimum pH at which all compounds will be extracted efficiently, the pH of the aqueous buffer (NaH_2PO_4) (donor

phase) was optimized between pH 2 and 10 using 3 samples per pH, a total of twenty seven (2 g) bovine kidney samples. The samples were fortified with spiking solution containing seven analytes as well as internal standards mixture and subjected to the HF-SLM procedure as described in 3.3.7 above. The pH of NaH_2PO_4 was adjusted with acetic acid to lower the pH and increased with sodium hydroxide solution. Other parameters; liquid membrane (triethylamine), extraction time (1 hour) and acceptor phase (0.1% formic acid) were held constant.

3.3.8.3 Optimization of pH of selected acceptor

The performance of 0.1% formic acid (pH 3.0), 0.1% acetic acid (pH 3.0) and 0.1 M hydrochloric acid (pH 3.0) as the acceptor phase was investigated. Nine 2 g bovine kidney samples were weighed, fortified with spiking solution containing seven analytes as well as internal standards mixture and subjected to the HF-SLM procedure as described in 3.3.7 above. Three samples were used with each acceptor phase. The liquid membrane (triethylamine), donor phase (NaH_2PO_4) and stirring time of 1 hour were kept constant during this experiment. The pH of the acid which gave good extraction efficiencies was further optimized between pH 2 and 6 by using 15 extracted samples and subjecting them to the hollow fiber supported liquid membrane procedure again as described in 3.3.7 above.

3.3.8.4 Effect of extraction (stirring time)

To determine extraction time of analytes, time was varied between 30 and 120 minutes to achieve an optimum extraction time. Optimization of extraction time was done using TEA as the liquid membrane, NaH_2PO_4 (pH 7) as the donor phase and 0.1 % formic acid (pH 3) as the acceptor phase.

Nine 2 g bovine kidney samples were weighed, fortified with spiking solution containing seven analytes as well as internal standards mixture and subjected to the HF-SLM procedure as described in 3.3.7 above. The samples were equally divided among the three extraction times.

3.3.9 Developing a dispersive solid phase extraction (dSPE) method

Another sample extraction/ clean-up method; dispersive solid phase extraction (dSPE) was developed as described below and later the performance merits of the two methods were evaluated. Ten millimeters of a mixture of water/acetonitrile (2:8 (v/v)) were added to the weighed 2 g tissue samples fortified with a spiking solution containing seven analytes as well as internal standards mixture. Samples were mechanically shaken with a multi reax mixer for 10 minutes followed by centrifugation at 5 °C, 4000 rpm, for 10 minutes. The supernatants were transferred into labeled 15 mL polypropylene centrifuge tubes containing 500 mg Sepra C18 SPE sorbent (BondElut) and vortexed for 5 minutes. The mixture was centrifuged again at 5 °C, 4000 rpm, for 5 minutes and 5 mL aliquot of the resulting supernatant was transferred into a test tube. The contents were evaporated down to 1 mL under a stream of N_2 using Turbo-vap LV with a water bath set at 40°C. The samples were then filtered through 0.45 µm PVDF membrane filters prior to injecting 20 µL into LC-MS/MS for analysis. The described procedure was adopted from Zhou et.al, Phenomenex applications note [98]. Figure 3.2 below shows a flow diagram for a typical dSPE method.

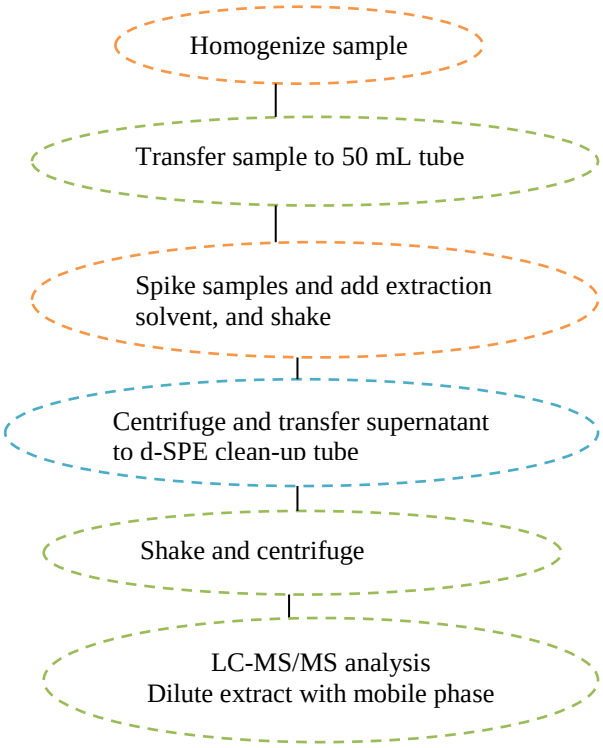


Figure 3.2: Flow diagram of dispersive solid phase extraction (dSPE) [99]

3.4 Method validation for HF-SLM and dSPE

Council directive 2002/657/EC guidelines describes parameters which were subjected to the HF-SLM and dSPE methods to validate their performance [13]. These parameters include; calibration function, identification, specificity, linearity, precision, accuracy and analytical limits. The precision of the method was evaluated in terms of repeatability (single day or intraday precision) and within-laboratory reproducibility (different days - interday precision). Analytical limits studied covered limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ). Validation was done for both HF-SLM and dSPE methods.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Mass spectral analysis

The optimized parameters of the seven quinolone compounds studied are as shown in Table 4.1 while Table 4.2 shows retention times and MRM transitions obtained for each analyte. Two MRM transitions were selected for each analyte. The most intense transition was used for quantification while the minor for confirmation (identification) of the analytes.

Table 4.1: Optimized MS parameters of each analyte

Analyte & Molecular weight (g mol^{-1})	**MS Parameters**					
	Q1 Mass (m/z)	**Q3 Mass (m/z)**	**Dwell time (msec)**	**DP (V)**	**CE (V)**	**CXP (V)**
Enrofloxacin 359.4	360	342	50	81	27	8
	360	316	50	81	37	20
	360	245	50	81	31	8
Ciprofloxacin 331.34	332	314	50	81	29	8
	332	288	50	81	27	8
	332	231	50	81	53	20
Difloxacin 399	400	356	50	101	29	14
	400	382	50	101	31	16
	400	299	50	101	37	8
Danofloxacin 357	358	340	50	86	31	20
	358	82	50	86	71	14
	358	314	50	86	27	8
Norfloxacin 319	320	302	50	66	29	18
	320	276	50	66	25	24
	320	233	50	66	35	18
Sarafloxacin 385.36	386	368	50	81	27	10
	386	342	50	81	33	10
	386	298	50	81	39	8
Nalidixic acid 232.24	233	215	50	46	21	18
	233	187	50	46	37	14
	233	104	50	46	59	8
Enrofloxacin-d5 359	365	347	50	61	29	30
	365	321	50	61	29	26

Analyte & Molecular weight (g mol^{-1})	**MS Parameters**					
	Q1 Mass (m/z)	**Q3 Mass (m/z)**	**Dwell time (msec)**	**DP (V)**	**CE (V)**	**CXP (V)**
Norfloxacin-d5 323	325	307	50	51	31	18
	325	231	50	51	55	12
Difloxacin-d3 399	403	385	50	61	33	22
	403	359	50	61	29	18

Table 4.2: MRM transitions, collision energies and retention times of each analyte

Analyte and Molecular Weight ($g\ mol^{-1}$)	**Retention time (t_R, min)**	**Internal Standard used**	**Quantification ion (Q1)(m/z)**	**Collision Energy (CE) (V)**	**Identifying Ion (Q3)(m/z)**	**Collision Energy (CE) (V)**
Enrofloxacin 359.4	4.40	ENR-d_5	360 → 342	27	360 → 316	37
Ciprofloxacin 331.34	4.28	ENR-d_5	332 → 314	29	332 → 288	27
Difloxacin 399	5.31	DIF-d_3	400 → 356	29	400 → 382	31
Danofloxacin 357	4.54	DIF-d_3	358 → 340	31	358 → 82	71
Norfloxacin 319	3.91	NOR-d_5	320 → 302	29	320 → 276	25
Sarafloxacin 385.36	5.75	DIF-d_3	386 → 368	27	386 → 342	33
Nalidixic acid 232.24	8.97	NOR-d_5	233 → 215	21	233 → 187	37
Enrofloxacin-d_5 359	4.34		365 → 347	29	365 → 321	29
Norfloxacin-d_5 323	3.78		325 → 307	31	325 → 231	55
Difloxacin-d_3 399	5.25		403 → 385	33	403 → 359	29

Key: ENR-d_5 Enrofloxacin-d_5; DIF-d_3 Difloxacin-d_3; NOR-d_5 Norfloxacin-d_5

4.2 Chromatographic separation

Despite testing several gradient elution programs and varying the pH of mobile phase using the Phenomenex Luna 3 μm C18, 150 mm x 2.0 mm, 100 Å column, the quinolones under study could not be resolved. Changing the stationary phase to an Agilent Zorbax Eclipse 1.8μm XDB-C18 50 mm x 4.6 mm column did not improve the separation. The separation greatly improved when the column was changed to Phenomenex Kinetex 2.6 μm XB-C18, 100 mm x 4.6 mm, 100 Å.

Under these conditions seven quinolones; (enrofloxacin, ciprofloxacin, danofloxacin, difloxacin, norfloxacin, nalidixic acid and sarafloxacin) and three internal standards (difloxacin-d_3, norfloxacin-d_5 and enrofloxacin-d_5) were separated (Figure 4.1). Separation of the ten analytes was finally achieved using a modified method from Phenomenex applications note [100]. A gradient method was developed using a Phenomenex Kinetex 2.6 μm XB-C18,100 mm x 4.6 mm, 100 Å column consisting of mobile phase A (0.1% formic acid in water) and B (methanol), at a flow rate of 0.4 mL min^{-1}, injection volume of 20 μL. Column temperature was optimized at 40℃. The developed separation method and the optimized LC-MS/MS conditions were used in the present study. Co-elution was still observed with some compounds such as enrofloxacin and ciprofloxacin due to the structural similarity of the two analytes. Good intensities for all analytes ranging from 1.0 x 10^5 for norfloxacin as the lowest to 9.3 x 10^5 for nalidixic acid as the highest, as well as good peak shapes were obtained. Internal standards were used in this study because they are useful in mass spectrometry during quantification. They are used to account for various method variables such as variability in extraction efficiency, matrix suppression, variability in injection volume, recovery during transfer of samples from one tube

to another as well as pipetting and clean-up stages [101]. It is normally added at the beginning of sample preparation before extraction to all samples, blanks and calibration standards at the same concentration level. During data analysis, peak ratios (i. e analyte concentration against internal standard concentration) are used in order to account for any loss of analytes that may have occurred at any stage during analysis because the same fraction of each is believed to be lost in any operation [102].

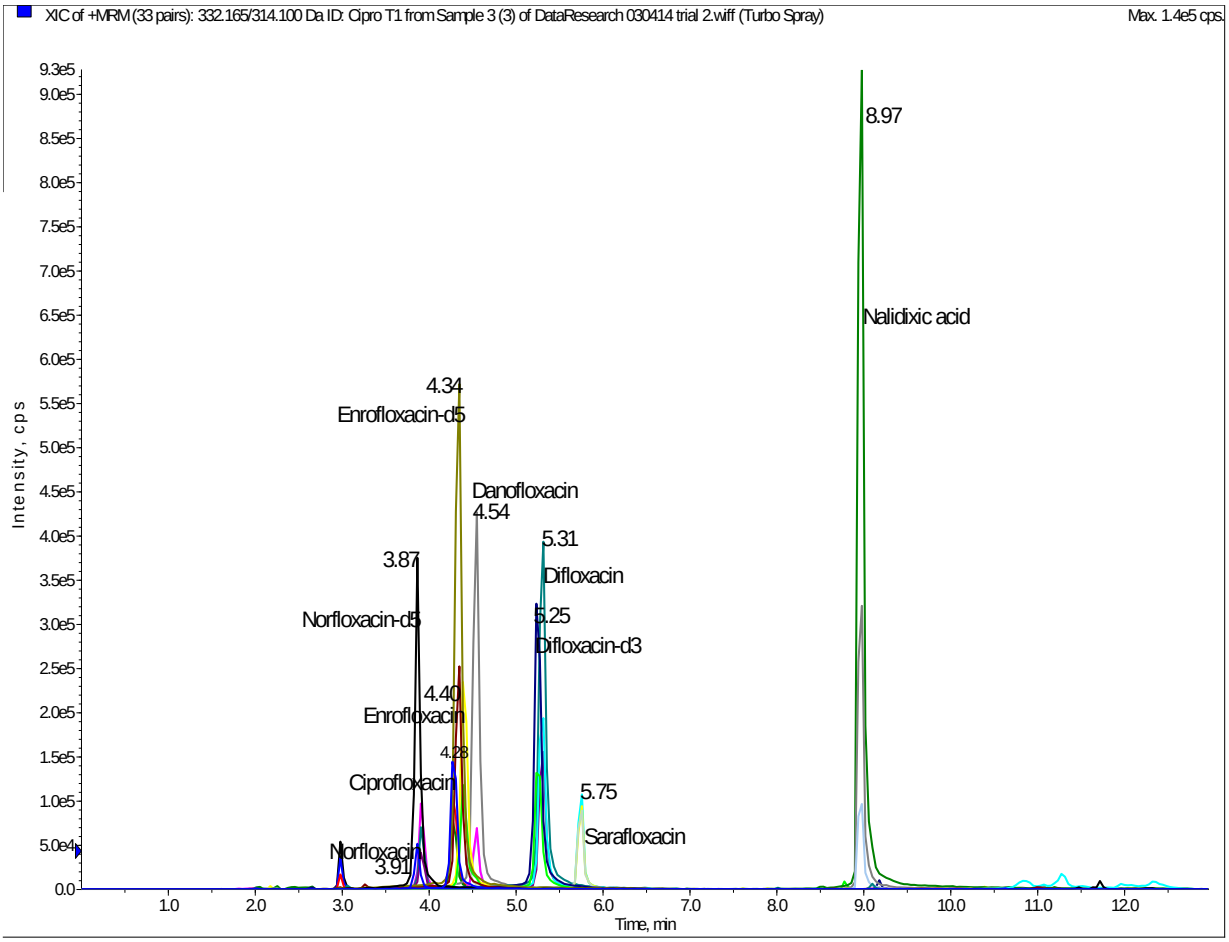


Figure 4.1 Chromatogram showing separation of 10 compounds (7 quinolones plus 3 internal standards) using Phenomenex Kinetex 2.6 µm XB-C18, 100 mm x 4.6 mm, 100Å. Mobile phase: A (0.1% formic acid in water) and B (methanol), at a flow rate of 0.4 mL min^{-1}, injection volume of 20 µL and column temperature was optimized at 40℃.

4.3 Optimization of hollow fiber- supported liquid membrane (HF-SLM)

A hollow fiber-supported liquid membrane method was developed and optimised for the extraction of quinolones from bovine kidney. The results are discussed in the next section below.

4.3.1 Selection of liquid membrane

In this study, a three-phase HF-SLM extraction system was used [66]. Selection of a liquid membrane is based on the following characteristics; (a) it should be immiscible with water and of low polarity to ensure that it remains within the pores of the fiber during extraction with no leakage to the aqueous sample, (b) less volatile to prevent evaporation and (c) low viscosity to ensure high and fast flow of analytes in one direction termed "rapid mass transfer" [15, 77]. Two solvents were investigated as possible liquid membrane; triethylamine (TEA) and diethyl ether (DEE). 0.1 % formic acid (pH 3.0) was used as an acceptor phase and NaH_2PO_4, as the donor phase at pH 7.0. Samples were stirred for 1 hour. Figure 4.2 compares the extraction efficiencies of seven quinolones when using triethylamine and diethyl ether as the liquid membranes. It is evident that five of the compounds, viz ciprofloxacin, danofloxacin, difloxacin, enrofloxacin and sarafloxacin were extracted better with triethylamine (% EF ranging from 88 – 118 %.) compared to diethyl ether which gave EFs of 37 to 109 %. The observed extraction efficiencies can be explained by the solubilities and hydrophobicities of the analytes. The solubility of sarafloxacin, difloxacin, nalidixic acid, enrofloxacin and norfloxacin in water at 25 °C are very low (i.e. 1, 5, 0.1, 0.15 and 0.28 [103, 104] mg mL^{-1} respectively, and therefore would be expected to be partitioned more into the organic phase. According to Sarafraz-Yazdi and Amiri, hydrophobic analytes are easily extracted into organic solvents from aqueous solutions [15], as observed with high

extraction efficiencies of sarafloxacin (110 %), difloxacin (118 %) and enrofloxacin (118 %) which are more hydrophobic. The opposite was observed for ciprofloxacin and danofloxacin which have high solubilities in water but did not extract well in diethyl ether. Both analytes extracted well in triethylamine with extraction efficiencies of 88 and 101 % respectively. The solubility for ciprofloxacin in water is 30 mg mL^{-1} at 20 °C while that of danofloxacin is 156.0 mg mL^{-1} at pH 5. Their good extraction efficiencies could have been enhanced by diffusion through a membrane solvent (triethylamine) which is less soluble in water (more hydrophobic) in comparison to diethyl ether (solubilities: 5.5 g 100 g^{-1} (25℃) for triethylamine [105] and 6.05 g 100 mL^{-1} (25℃) for diethyl ether). Nalidixic acid and norfloxacin were extracted more favorably in diethyl ether with extraction efficiencies of 98 and 97 % respectively as compared to 57 and 56 % respectively in triethylamine. These can be explained by that though both nalidixic acid and norfloxacin have low solubility in both water and ethers (0.1 and 0.01mg mL^{-1} [104] respectively), that is more hydrophobic, back extraction or diffusion seemed to be less in diethyl ether than in triethylamine leading to them being extracted more in diethyl ether. Overall triethylamine was selected as the liquid membrane for all the work as it gave better results for most analytes than diethyl ether and also because it is more hydrophobic compared to diethyl ether.

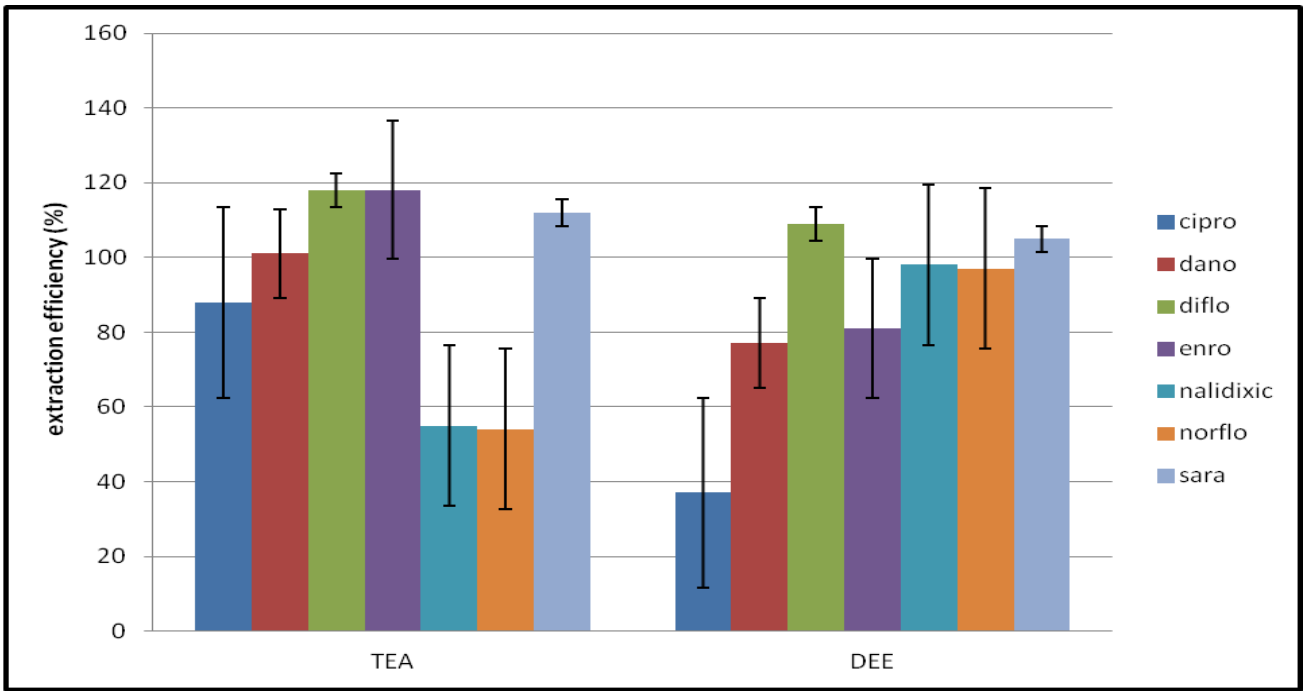


Figure 4.2: Selection of liquid membrane; TEA (triethylamine); DEE (Diethyl ether); NaH_2PO_4 pH 7 (donor phase), 0.1 % formic acid (acceptor phase), 60 minutes stirring time.

4.3.2 Effect of pH of donor phase

To select an optimum pH at which all compounds will be extracted efficiently, the pH of donor phase has to be optimised such that pH > pKa + 3.3 [106]. The principle of HF-SLM requires that analytes in the donor phase be in a neutral state (around pKa values) for them to diffuse through the supported liquid membrane into the acceptor phase [106, 107]. The pKa values of quinolones ranges from 6 to 9 (Table 2.1) making them neutral between pH 7 to 8. The pH of NaH_2PO_4 (donor phase) was therefore varied between 2 and 10. Triethylamine was used as a liquid membrane, 0.1 % formic acid (pH 3.0) as an acceptor phase and stirring time of 1 hour. Figure 4.3 shows the extraction efficiencies obtained with variation of pH of the donor phase. The extraction efficiencies increased from pH 2 up to pH 7 and then decreased under alkaline conditions. The obtained extraction efficiencies for all compounds

at pH 7 ranged between 89 - 116 %. Ciprofloxacin was not extracted at all at pH 3. According to literature, it has been found that most quinolones have four ionisable functional groups, one carboxylic group and three basic nitrogen sites (Table 2.1) leading to four pK_a values. Ciprofloxacin has four pK_a values (3.01, 6.14, 8.7 and 10.58) [40]. Thus at pH 3 of the donor phase (which was equal to that of the acceptor phase) ciprofloxacin could not be extracted at all, because there was no driving force by the proton gradient which is required to transport analytes in one direction (from the donor to acceptor phase) since the pHs were equal. The pK_a of other analytes are greater than 3, therefore at pH 3, they were able to be extracted into the acceptor phase. For analytes to be extracted the pH of the sample must be adjusted to a value sufficiently high such that the analytes are uncharged, and can be extracted into the liquid membrane [108]. A similar trend was observed and reported by Poliwoda et. al when they studied the influence of donor phase pH on four flouroquinolones using HF-SLM. The enrichment factors were observed to increase from pH 2 until they reached a maximum at pH 6 then decreased [79] similar to what was observed in this study. It has been observed in literature that higher pH values (i.e. pH 10) resulted in membrane destabilization [79] which has been observed with norfloxacin and sarafloxacin under this pH since they were not extracted at all. For the rest of the study pH 7 was chosen as the optimum for the donor phase since it gave superior extraction efficiencies for all the analytes.

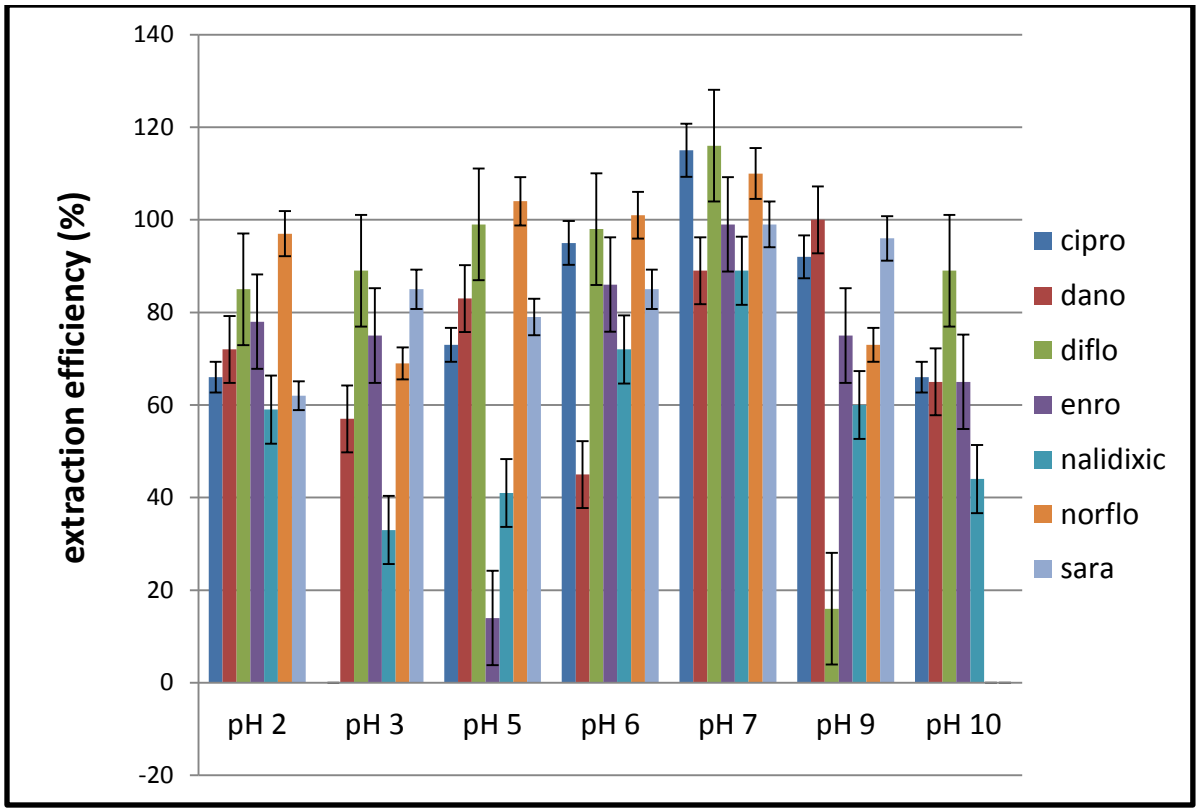


Figure 4.3: Optimization of pH of NaH_2PO_4 (donor phase) from pH 2 to 10; triethylamine (liquid membrane); 0.1 % formic acid (acceptor phase); 60 minutes stirring time

4.3.3 Effect of pH of selected acceptor phase

Three acids were investigated as possible acceptor solutions. Acids were used in this case so as to enhance extraction of basic analytes from the organic (liquid membrane) phase into the acceptor solution. Figure 4.4a shows the results from all three acids which gave reasonably good extraction efficiencies (76 -113 %) for all analytes except nalidixic acid which was less extracted in acetic and hydrochloric acids with extraction efficiencies of 10 and 38 % respectively. Nalidixic acid is structurally different from other quinolone antibiotics, it is also more acidic with a pKa of 5.95 [43]. In HF-SLM, nalidixic acid will extract well with a basic acceptor phase of a higher pH (i.e pH >10)

than a lower pH. Formic acid (0.1 %) was then chosen as the optimum acceptor phase and also for its compatibility with LC-MS/MS. Formic acid was further investigated by varying the pH in the range of 2 to 6 to obtain the optimum condition. The extraction efficiencies obtained are shown in Figure 4.4b. It can be observed that at pH 3 extraction efficiencies ranging from 85 – 112 % were obtained for all analytes. This can be explained by the concept of mass transfer (net diffusion across the membrane or phase), whereby in order to obtain a high mass transfer the presence of proton gradient between the acceptor and donor phase is essential to ensure that transport of analytes goes only in one direction. The proton or concentration gradient is responsible for the driving force of the process [79, 107, 109]. Thus the pH of the acceptor phase (i.e. 0.1 % formic acid, pH 3) should be lower than that of the donor phase (i.e. NaH_2PO_4, pH 7), to enhance extraction of analytes in one direction; since a higher pH in the donor phase suppresses solubility of the analytes whereas a lower pH in the acceptor phase promotes solubility and this enhances extraction of basic compounds into the membrane solvent and further into the acceptor phase without back-extraction to the membrane solvent [15].

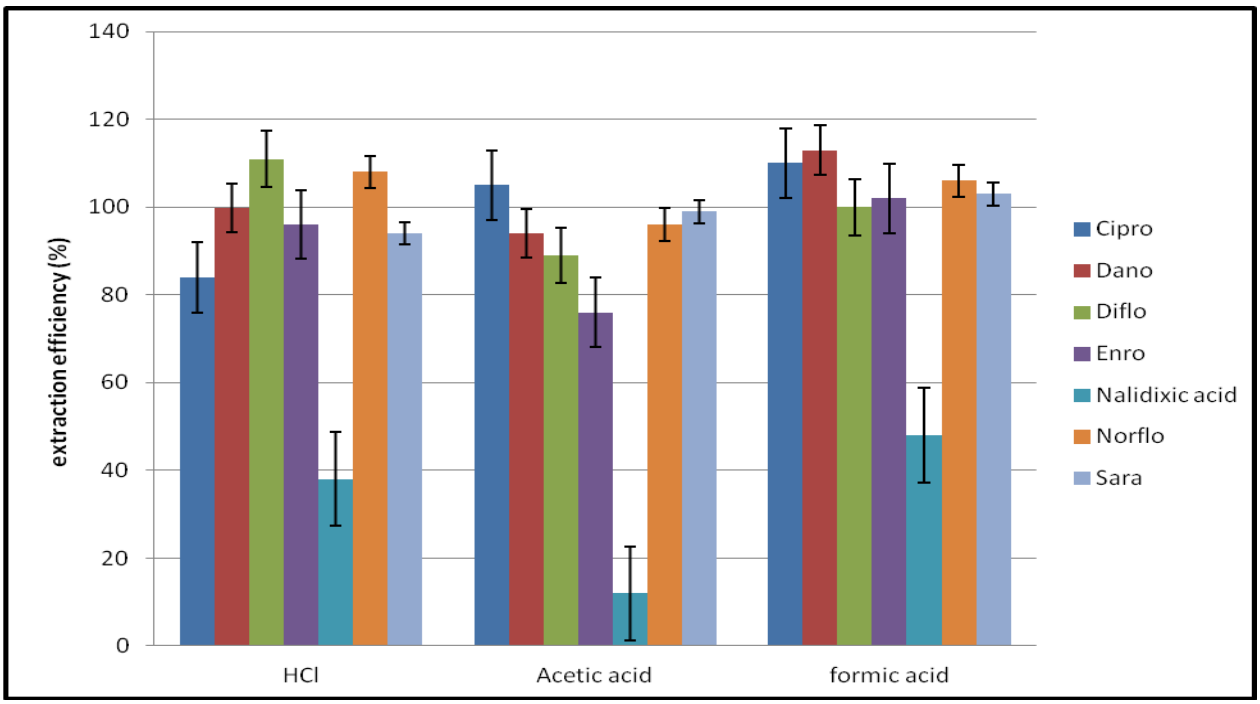


Figure 4.4a: Selection of acceptor phase (i) 0.1 M HCl (ii) 0.1% acetic acid (iii) 0.1% formic acid; triethylamine (liquid membrane), NaH_2PO_4 pH 7 (donor phase), 60 minutes stirring time

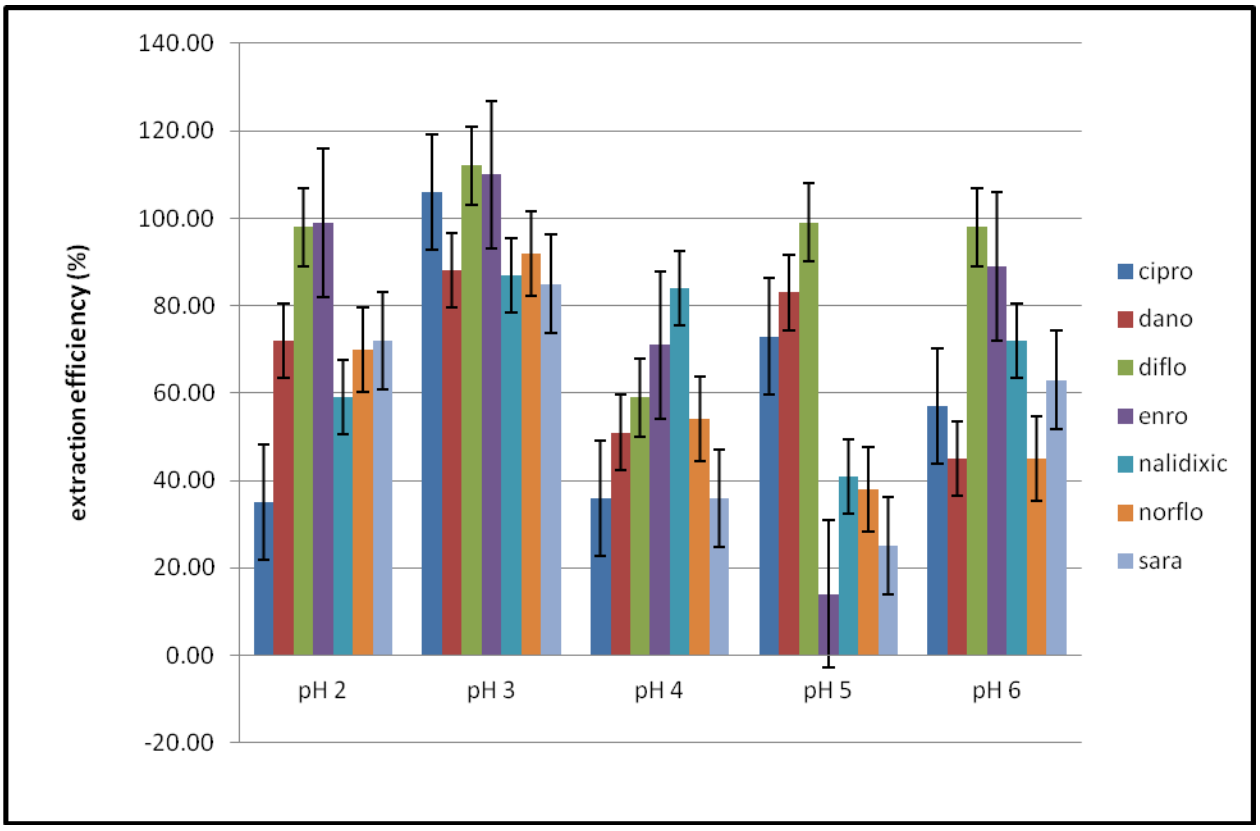


Figure 4.4b: Optimization of pH of selected acceptor phase (0.1% formic acid) between pH 2 to 6; triethylamine (liquid membrane), NaH_2PO_4 pH 7 (donor phase), 60 minutes stirring time.

4.3.4 Evaluation of extraction time

To determine the total extraction time of analytes, a principle of "equilibrium extraction through membrane" [66] led to varying the extraction time from 30 to 120 minutes to achieve an optimum extraction time since extraction of analytes through the membrane is time dependent. Optimization of extraction time was carried out using TEA as the liquid membrane, 0.1% formic acid as the acceptor phase at pH 3.0 and NaH_2PO_4 pH 7 as the donor phase. From the results in Figure 4.5, it can be

observed that extraction efficiencies increased with time from 30 to 60 minutes, then decreased between 60 and 120 minutes. The observed trend can be perfectly backed up by the statement by Ho et.al which states that "LPME is an equilibrium extraction technique, and the recovery increases rapidly with increasing extraction time up to a certain level where the recovery versus time gradually decreases and the extraction system enters an equilibrium state" [77]. Another concept from Jönsson .et. al. also explains the trend observed in terms of concentration profile in the donor channel being constant with time also leading to constant extraction efficiency and a linear increase of the concentration in the acceptor channel with time [107] which both shows complete trapping of analytes in the acceptor channel. These conditions are obviously desirable in practical application of the technique. With incomplete trapping, the extraction efficiency will decrease with time until a sufficient concentration in the acceptor channel is reached, representing equilibrium concentrations of the analyte in all three phases and, consequently, zero flux [107]. Most analytes were well extracted within 60 minutes with extraction efficiencies ranging from 55 -118 %. This time (i.e. 60 minutes) was therefore selected as the optimum and used for the rest of the investigations in this work. The HF-SLM optimum conditions were therefore; NaH_2PO_4 at pH 7 as the donor phase, triethylamine as the liquid membrane, 0.1 % formic acid at pH 3 as the acceptor phase, and extraction time of 60 minutes. These optimum conditions were used for validation and applicability of the method to real samples.

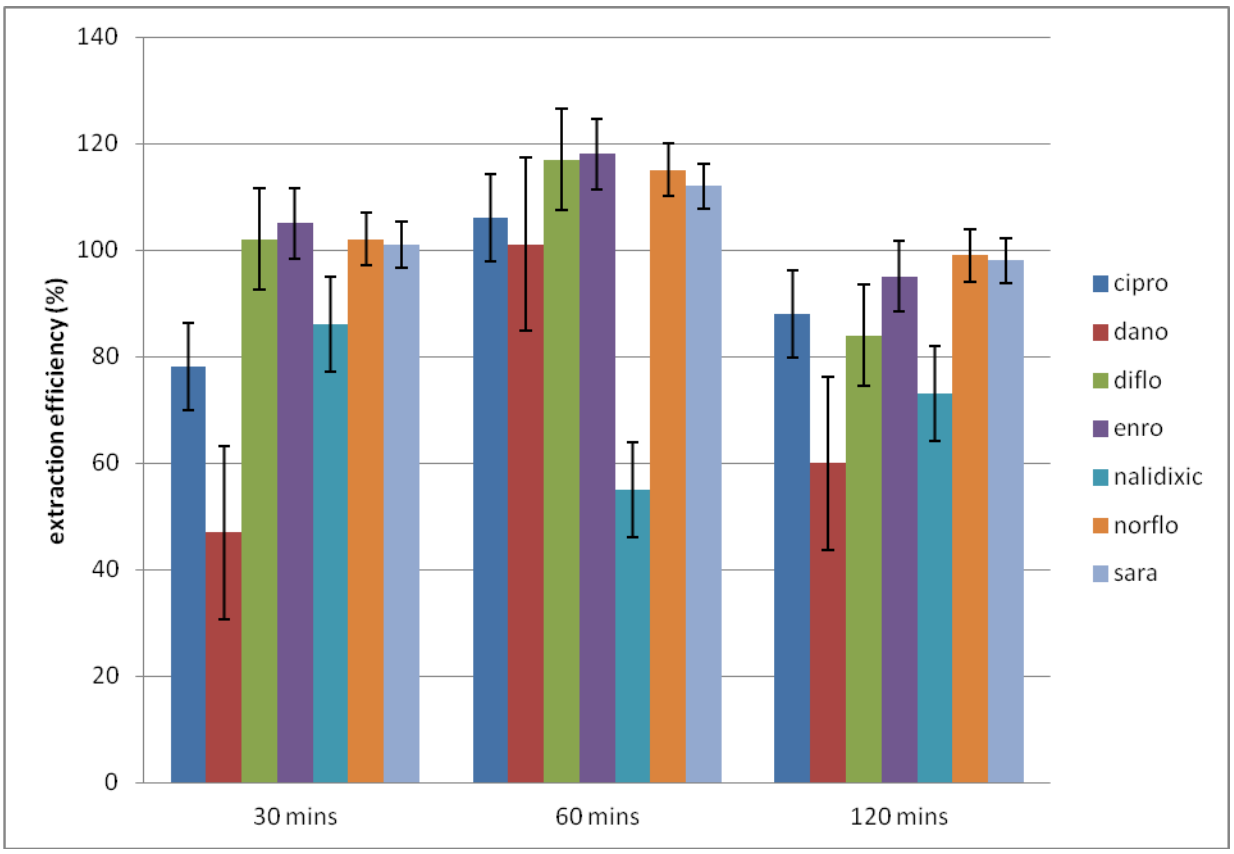


Figure 4.5: Effect of extraction time (i) 30 minutes (ii) 60 minutes (iii) 120 minutes; triethylamine (liquid membrane), NaH_2PO_4 pH 7 (donor phase), 0.1 % formic acid pH 3 (acceptor phase).

4.4 Method validation for HF-SLM

4.4.1 Linearity

The linearity of the method was evaluated using a nine point matrix-matched calibration curve constructed by spiking a blank kidney sample with quinolones standard solution at 0, ¼, ½, ¾, 1, 1.5, 1.75, 2.0 and 2.5 MRL or action level (i.e. covering a concentration range of 12.5 - 1000 µg kg^{-1} for all analytes). Each

concentration level had nine replicates. Coefficients of determination (r^2) for all curves were greater than 0.97 (Table 4.3). This demonstrates linearity of the detector response over the concentration range analysed and gives confidence in the ability of the method to quantitate.

4.4.2 Limit of detection (LOD) and limit of quantification (LOQ)

The HF-SLM was validated for the limit of detection (LOD) and the limit of quantification (LOQ). The LOD is the lowest quantity of a substance that can be distinguished from the sample in the absence of that substance (a blank value) within a stated confidence limit (generally 1%) while limit of quantification (LOQ) is the limit at which we can reasonably tell the difference between two different values [13] or the smallest analyte concentration that can be quantified with a given confidence level. Both limits can be determined by the standard error approach using the calibration curve [110]. According to this approach the limit of detection is defined by;

$$\mathbf{LOD = 3.3*(S_y/\mathit{m})} \qquad \mathbf{(1)}$$

and the limit of quantification is defined by

$$\mathbf{LOQ = 10*(S_y/\mathit{m})} \qquad \mathbf{(2)}$$

Where $\boldsymbol{m}$ is the slope of the calibration curve and $\boldsymbol{S_y}$ is the standard error of the calibration curve given by the equation below;

$$S_y = \sqrt{\sum \frac{(Y_i - mx_i - b)^2}{n - 2}} \quad (3)$$

Where: Y_i is the y value, X_i is the x value, b is the y intercept, m is the slope and n is number of the degrees of freedom [13].

Alternatively, LOD and LOQ can be calculated using the standard deviation of concentration of seven blank samples at the lowest calibration level using the formulas below:

$$\mathbf{LOD = Conc_{blank} + 3\ (SD_{blank}/lowest\ standard).} \quad \mathbf{(4)}$$

$$\mathbf{LOQ = Conc_{blank} + 10\ (SD_{blank}/lowest\ standard).} \quad \mathbf{(5)}$$

Where; $\mathbf{Conc_{blank}}$ is the concentration of the blank sample, $\mathbf{SD_{blank}}$ is the standard deviation of the blank, **3** is the signal-to-noise ratio for limit of detection, and **10** is the signal-to-noise ratio for limit of quantification.

Equations (1), (2) and (3) were used to calculate LOD and LOQ. Table 4.4 shows LOD and LOQ values obtained in this work which ranged from 3 to 39 µg kg^{-1} and 11 to 130 µg kg^{-1} respectively. These values are much higher for some analytes in comparison to data reported by other authors. Examples in literature where similar analytes have been determined in distilled water, surface water, wastewater and bovine urine using HF-SLM with HPLC obtained the following LODs; ciprofloxacin 3 ng L^{-1} (LOD in distilled water) and 10 ng L^{-1} (LOD in surface water and urine samples); danofloxacin 5 ng L^{-1} (LOD in distilled water) ,7 ng L^{-1} (LOD in urine samples) and 13 ng L^{-1} (LOD in surface water); enrofloxacin 7 ng L^{-1} (LOD in urine samples,13 ng L^{-1} (LOD in distilled water) and 25 ng L^{-1}

(LOD in surface water) [7, 79]. This trend is expected since water matrices are less complex compared to biological matrix such as bovine kidney leading to higher detection and quantification limits. Some researchers employed solid phase extraction in swine kidney followed by LC-MS/MS. The LODs reported were not significantly different for the same analytes studied (i.e. ciprofloxacin <15 µg kg^{-1}; enrofloxacin <10 µg kg^{-1}; nalidixic acid <10 µg kg^{-1}; norfloxacin <10 µg kg^{-1} [50]) while for danofloxacin the value differed significantly (i.e. danofloxacin <10 µg kg^{-1} [50]). The LODs and LOQs obtained for quinolones extracted by liquid-liquid extraction from chicken muscle and followed by LC-MS/MS were as low as (0.2 - 1.5) and (0.7 – 5.0) µg kg^{-1} respectively for ciprofloxacin, danofloxacin and enrofloxacin [94]. The observation made with regard to the low LOD and LOQ values in the paper by Chiaochan et.al [94] was that the linearity range used was lower (1 -100 µg kg^{-1}) compared to the linearity range used in this study which ranges from 12.5 as the lowest to 1000 µg kg^{-1} as the highest, for the various analytes as shown in Table 4.3. Linearity range influences the values of LODs and LOQs in that they can be calculated based on the response obtained from the calibration curve to estimate the standard error, then dividing the error with the slope of the curve, multiplied by 3 (LOD) or 10 (LOQ) signal-to-noise ratio. The response for a calibration curve with a lower linearity range will be lower, leading to low LOD and LOQ and vice versa. These limits can also be estimated by analysing spiked blank sample at the lowest calibration level and determining the concentration of each analyte which provided signals with S/N equal to 3 and 10 respectively as was the case in the paper by Chiaochan et. al. Higher linearity ranges used in this study were based on the MRL/action levels of the analytes as stipulated in EU directives.

Table 4.3: Summary of the validated parameters

Compound	Linear range, (µg kg^{-1})	Regression equation	R^2	LOD, (µg kg^{-1})	LOQ, (µg kg^{-1})
CIPRO	50-250	y = 0.00184x + (-0.0115)	0.9929	17	58
ENRO	50-250	y = 0.00346x + 0.0249	0.9781	12	41
DANO	100-500	y = 0.00755x + (-0.0479)	0.9955	28	95
DIFLO	200-1000	y = 0.00262x + (-0.00584)	0.9975	39	130
NORFLO	25-125	y = 0.00212x + (-0.0162)	0.9714	4	13
NALI	12.5-62.5	y = 0.0972x + 0.2070	0.9888	3	11
SARA	50-250	y = 0.00368 + (-0.00506)	0.9910	18	61

4.4.3 Precision study

Precision was estimated by fortifying blank kidney samples at three different levels (0.5, 1.0 and 1.5 times the MRL) in seven replicates at each level for three days. Repeatability (within batch) was calculated as the relative standard deviation (RSD) of results obtained for each analyte at each level after replicates (n = 7) were analysed under the same conditions, same operator, and on the same day. The RSD was calculated from the mean concentration of each batch. The RSDs obtained ranged from 1 – 23% which fall within the recommended limits of ≤ 23% as stipulated by the European Commission 2002/657/EC (Table 4.4). This shows that the method has good repeatability.

Two analysts were involved for within laboratory reproducibility. Moreover, for the mobile phase and extraction purposes, different batches of acetonitrile and methanol were used. Within –laboratory reproducibility was calculated as %RSD of analysis done at two concentration levels (0.5 MRL and 1.0 MRL) over a period of three days. The RSDs obtained also ranged from 2 – 15% which fall within

the recommended limits of ≤ 23% as stipulated by the European commission 2002/657/EC (Table 4.5) showing that the method is reproducible.

Table 4.4: Intraday precision of HF-SLM method

Analyte	**MRL/action level (**µg kg^{-1})	**Mean $C_{calculated}$** (n = 7)	**SD**	**%RSD**
CIPRO	100	99	11.6	11.7
ENRO	100	96	7.44	7.75
DANO	200	191	24.7	12.9
DIFLO	400	412	17.5	4.25
NALI	25	27	3.76	13.9
NORFLO	50	53	7.61	14.4
SARA	100	99	7.42	7.49

Note: $C_{calculated}$- calculated concentration (µg kg^{-1})

Table 4.5: Reproducibility of HF-SLM

Analyte	Mean conc. at MRL/action level (obtained in 3 days)					
	Day 1 (Batch)	Day 2 (Batch)	Day 3 (Batch)	Mean Conc (n = 3)	SD	% RSD
	(n = 7 for each day)					
CIPRO	99	101	102	101	1.53	1.51
ENRO	96	112	102	103	8.08	7.8
DANO	191	184	204	193	10.1	5.23
DIFLO	412	388	371	390	20.6	5.28
NALI	27	24	27	26.0	1.73	6.65
NORFLO	53	54	50	52.5	2.08	3.98
SARA	99	100	101	100	1	1

Note: Conc. - concentration

4.4.4 Accuracy

Accuracy is determined using a Certified Reference Material (CRM). Unfortunately, there was no CRM available in this study. To determine trueness, accuracy was evaluated by spiking samples with standard solutions and calculating the recoveries. Seven replicates of bovine kidney samples were spiked at three levels (0.5, 1 and 1.5 MRL) (n = 21). The recoveries were calculated from the peak ratios obtained from a spiked matrix-matched calibration curve. Table 4.6 shows mean % recoveries for all quinolone analytes at three spiking levels (0.5, 1 and 1.5 MRL) using HF-SLM. Recoveries for all the analytes at the three concentration levels were in the range of 89-107 % which fall within the recommended acceptable range of 80-110 % as stipulated in EU commission 2002/657/EC.

Table 4.6: Recoveries (%) of extracted quinolones from bovine kidney at ½, MRL and 1.5 MRL spiking levels. (n = 21)

Analyte	**HF-SLM % Mean recovery**			
	½MRL	MRL	1.5MRL	SD
CIPRO	95	91	100	4.51
ENRO	106	103	107	2.21
DANO	98	89	101	6.51
DIFLO	100	100	100	0
NALI	100	104	99	2.61
NORFLO	95	105	98	5.17
SARA	100	100	94	2.28

4.4.5 Decision limit (CCα) and Detection capability (CCβ)

Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of (α = 95%) that a sample is non-compliant, as defined in the European Union Commission Decision 2002/657/EC directive [13]. CCα can be defined by the following equation;

$$CC\alpha = C_{MRL} + 1.64SD_{(at\ MRL)} \qquad (6)$$

which states that; the corresponding concentration at the permitted limit plus 1,64 times the standard deviation of the within-laboratory reproducibility equals the decision limit (α = 5 %) from the plotted curve.

On the other hand, detection capability **(**CCβ**)** also as defined in the European Union Commission Decision 2002/657/EC directive; means the smallest content of the substance that may be detected, identified or quantified in a sample with an error probability of (β = 95%). This means that the

detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of (β = 5%) [13]. The CCβ is therefore defined by the equation;

$$CC\beta = CC\alpha + 1.64SD_{(at\ CC\alpha)} \quad (7)$$

The decision limits (CCα) and detection capability (CCβ) were calculated using calibration curve procedure according to ISO 11843 [111]. Seven replicates of a blank kidney sample were fortified around the MRL in equidistant steps (0.5, 1.0, and 1.5 MRL). Samples were analysed and after identification calibration curves were plotted (peak ratio against concentration). Table 4.7 shows values obtained for CCα ranging from 28 µg kg^{-1} to 422 µg kg^{-1} and CCβ ranging from 29 µg kg^{-1} to 454 µg kg^{-1}. These values are useful for interpretation of results by qualifying the results as compliant or non-complaint. For example, if the results for enrofloxacin (MRL – 100 µg kg^{-1}) obtained for a certain sample is found to be 127 µg kg^{-1}, the results will be interpreted as non-compliant since the result is above the validated CCβ (125 µg kg^{-1}). If the value is above CCα (114 µg kg-1) but below CCβ, the results are interpreted as false positive and are compliant.

Table 4.7: CCα and CCβ of quinolones obtained with HF-SLM method.

Analyte	MRL/ action level (µg kg^{-1})	Mean C_{MRL} (µg kg^{-1})	SD within –lab reproducibility	CCα (µg kg^{-1})	CCβ (µg kg^{-1})
	n=7				
CIPRO	100	101	15	126	150
ENRO	100	103	7	114	125
DANO	200	193	5	201	210
DIFLO	400	391	19	422	454
NALI	25	26	1	28	29
NORFLO	50	52	1	54	56
SARA	100	100	0	123	146

Note: C_{MRL} – concentration at MRL level

4.5 Method validation for dSPE

Validation for dSPE was done following the same procedure used for validation of HF-SLM method.

Table 4.8 shows the summary of all the validated parameters for dSPE method.

Table 4.8: Summary of validated parameters for dSPE method

Analyte (MRL/ action level)	Validated parameters							
	% mean recovery at MRL/ action level	r^2	LOD	LOQ	Reprod (% RSD) at MRL/ action level	Repeat (% RSD) at MRL/ action level	CCα ($\mu g\ kg^{-1}$)	CCβ ($\mu g\ kg^{-1}$)
Cipro (100)	106	0.9969	12	40	22	13	127	149
Enro (100)	103	0.9986	10	33	7	2	107	111
Dano (200)	96	0.9963	37	74	17	6	218	227
Diflo (400)	114	0.9735	39	130	25	21	429	464
Nali (25)	100	0.9955	3	10	1	1	27	30
Nor (50)	101	0.9966	3	10	5	5	58	66
Sara (100)	110	0.9811	6	19	17	5	117	125

The LOD values obtained for ciprofloxacin, enrofloxacin, nalidixic acid and norfloxacin were comparable to the values obtained in a study for determination of flouroquinolones in swine kidney using solid phase extraction analysed by LC-MS/MS [50]. The LOD for danofloxacin is not comparable since the values differ by a magnitude of 3^3. Difloxacin and sarafloxacin were not studied in the same paper.

4.6 Comparison of HF - SLM with dSPE using student t-test

From the validation data obtained for the two methods, it can be noted that both methods gave good recoveries ranging between 89 – 114 % for all analytes studied, though the recovery for difloxacin (114% when using dSPE) went beyond the acceptable limit of 80-110 % as set in the EU commission 2002/657 EC. The obtained LODs are comparable between the two methods for some analytes (i.e. ciprofloxacin, enrofloxacin, difloxacin, norfloxacin and nalidixic acid) since they differ by a difference ranging between 0 - 12 µg kg^{-1}. This close agreement in LOD values was also observed in a paper by van Vyncht on analysis of fluoroquinolones in swine kidney using Solid Phase Extraction analysed by LC-MS/MS [50]. The same cannot be said for HF-SLM method since the LOD values obtained for quinolones extracted from water matrices analysed by HPLC [79], were lower in most cases than the ones obtained in this study. The LOQ values of the two methods differ for most analytes except for difloxacin, nalidixic acid and norfloxacin. In terms of precision, HF-SLM method has been found to be more precise for all analytes since very low % RSD (CV) were obtained (i.e. 0.66 -14.4 %). The precision for dSPE method was varying between analytes ranging from 1 - 25 %, with difloxacin going beyond the acceptable limit of ± 23%.

Statistical comparison using a two – tailed test at 95 % confidence interval was carried out using the mean recoveries at MRL/action level obtained from both methods to evaluate if there is any significant difference between the two methods. The obtained results show that there is a significant difference for ciprofloxacin, difloxacin and sarafloxacin because the calculated *t*-values are higher (11.0 – 26.7) than the *t*-critical values (4.30). On the other hand the t-values obtained for enrofloxacin, danofloxacin,

nalidixic acid and norfloxacin are less than (0 - 3.89) the *t*-critical value showing that there is no significant difference between the two methods during extraction of these analytes. The values are as shown in Table 4.9 using the student t-test at 95 % confidence interval [112].

Table 4.9: Comparison of HF - SLM with dSPE using student t-test for the mean recoveries of each quinolone analyte at MRL/action level (25 - 750 µg kg^{-1}) (n = 21)

Analyte	**Mean % recoveries HF-SLM**	**Mean % recoveries dSPE**	**Standard deviation**	***t* –value**	***t*-critical**
Ciprofloxacin	91	106	10.6	12.2	4.30
Enrofloxacin	103	103	0	0	4.30
Danofloxacin	89	96	4.95	3.89	4.30
Difloxacin	100	114	9.90	11.0	4.30
Nalidixic acid	104	100	2.83	1.68	4.30
Norfloxacin	105	101	2.83	1.68	4.30
Sarafloxacin	100	110	7.07	26.7	4.30

4.7 Application of HF-SLM to real bovine kidney samples

Some bovine kidney samples obtained from Botswana National abattoirs were analysed for quinolones using the optimized HF-SLM parameters. Ten bovine kidney samples were homogenized and weighed. Two samples were treated as quality control samples and spiked at MRL level. Five milliliters of a mixture of 0.1% phosphoric acid: acetonitrile (30:70 v/v) was added to all samples followed by 10 mL of NaH_2PO_4 at pH 7 to extract the analytes. The samples were applied to HF-SLM for further extraction and pre-concentration. A matrix-matched calibration curve was used for

quantification of the analytes in the samples with the addition of internal standards to all samples including the calibration curve. None of the analysed samples were positive for quinolones at detectable levels. Figure 4.6 shows a chromatogram from a negative sample, showing only peaks for the internal standards.

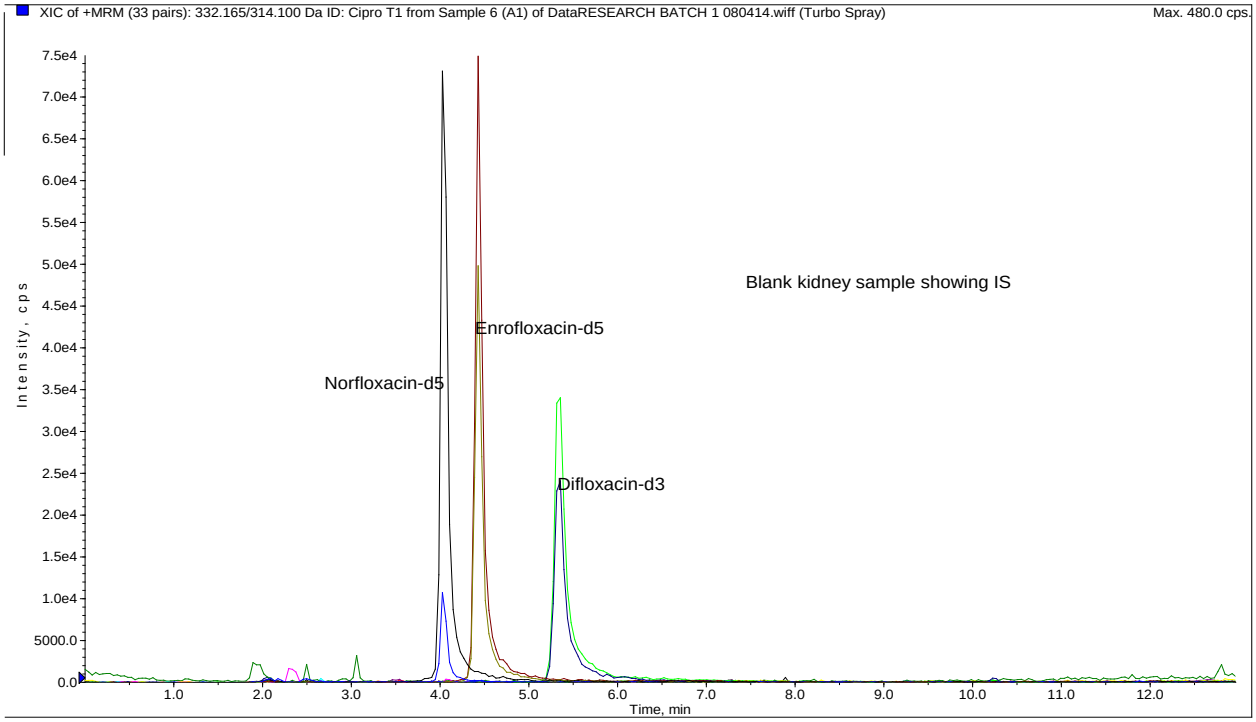


Figure 4.6 Chromatogram of extracted bovine kidney sample showing internal standards (enrofloxacin-d5, difloxacin-d3 and norfloxacin-d5) only.

Figure 4.7a below shows a chromatogram from a spiked quality control sample obtained using the optimized HF-SLM method. The obtained peaks in the chromatogram are not well resolved. Figure 4.7b to 4.7d shows the individual analytes from chromatogram 4.7a, which have been successfully extracted from the sample. The intensity of compounds obtained from HF-SLM extracted from spiked bovine kidney samples were lower for each analyte compared to those extracted with dSPE. For

instance, the intensities for enrofloxacin are 3.8 x 10^3 for HF-SLM and 2.5 x 10^5 for dSPE, for norfloxacin the values are 3.0 x 10^3 for HF-SLM while for dSPE is 1.0 x 10^5 (Figure 4.7b). HF-SLM is a delicate technique since the fibers used are very small and must be handled with care. Possibility of loss of analyte during extraction is very high which could have lead to the lower intensities.

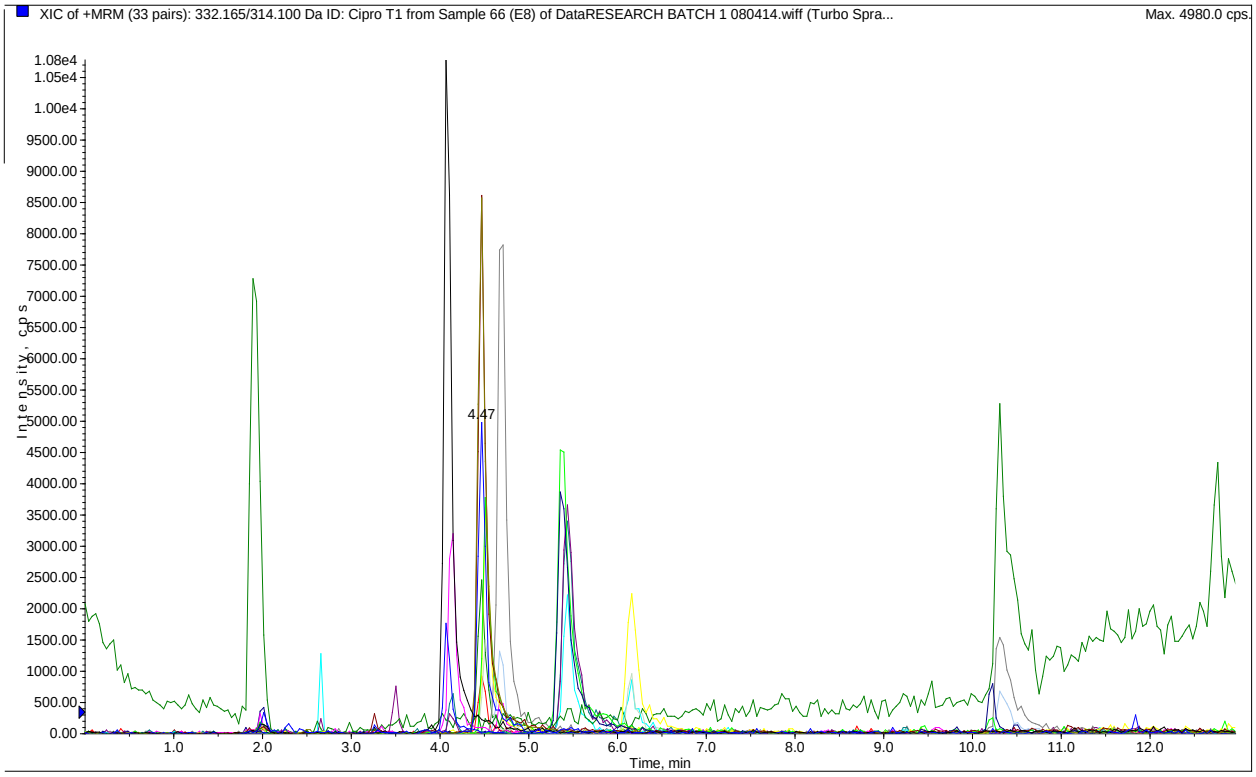


Figure 4.7a: Chromatogram showing separation of quinolones extracted from a spiked bovine kidney using the optimised HF- SLM method. A Phenomenex Kinetex 2.6 µm XB-C18 100 mm x 4.6 mm, 100Å column was used. Mobile phases A (0.1% formic in water) and B (methanol), at a flow rate of 0.4 mL min^{-1}, injection volume of 20 µL and column temperature was optimized at 40℃.

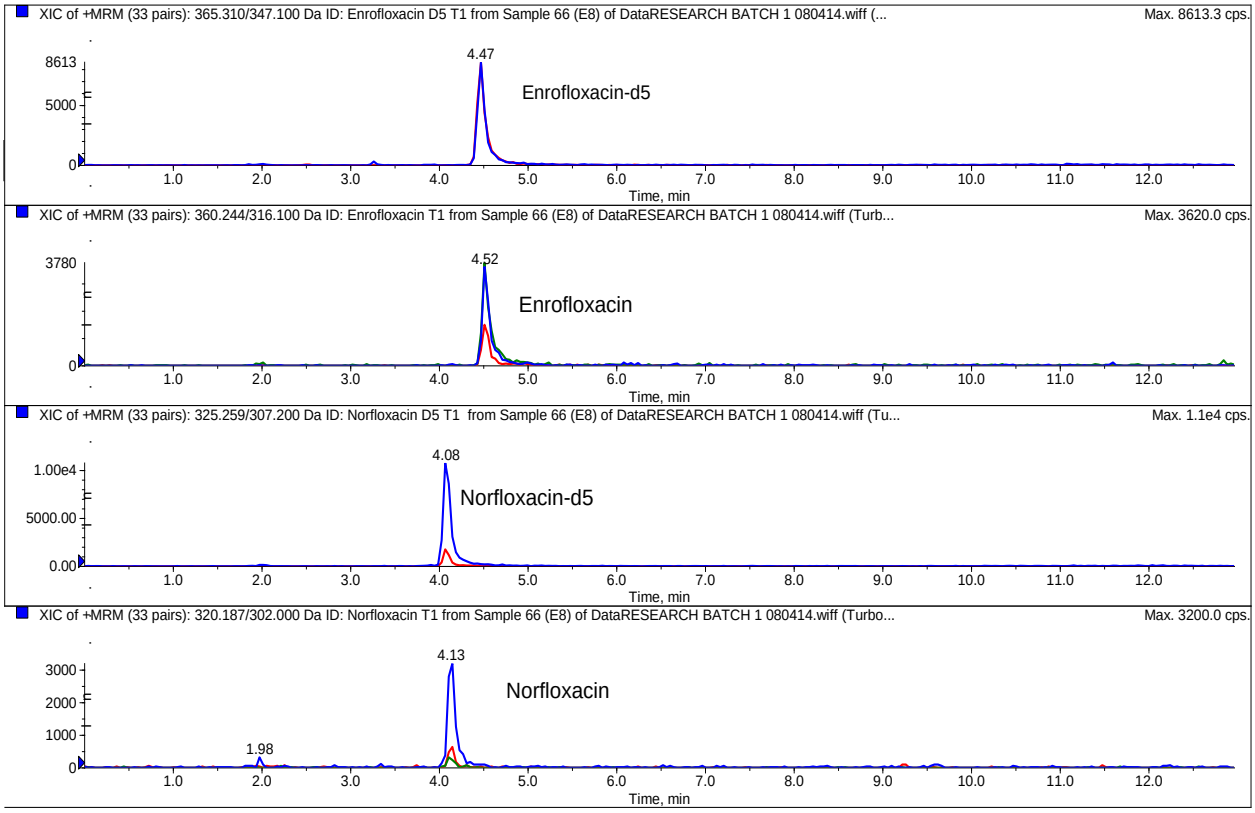


Figure 4.7b: Extracted ion chromatograms of each analyte obtained from the total ion chromatogram in Figure 4.7a; showing retention times of each analyte.

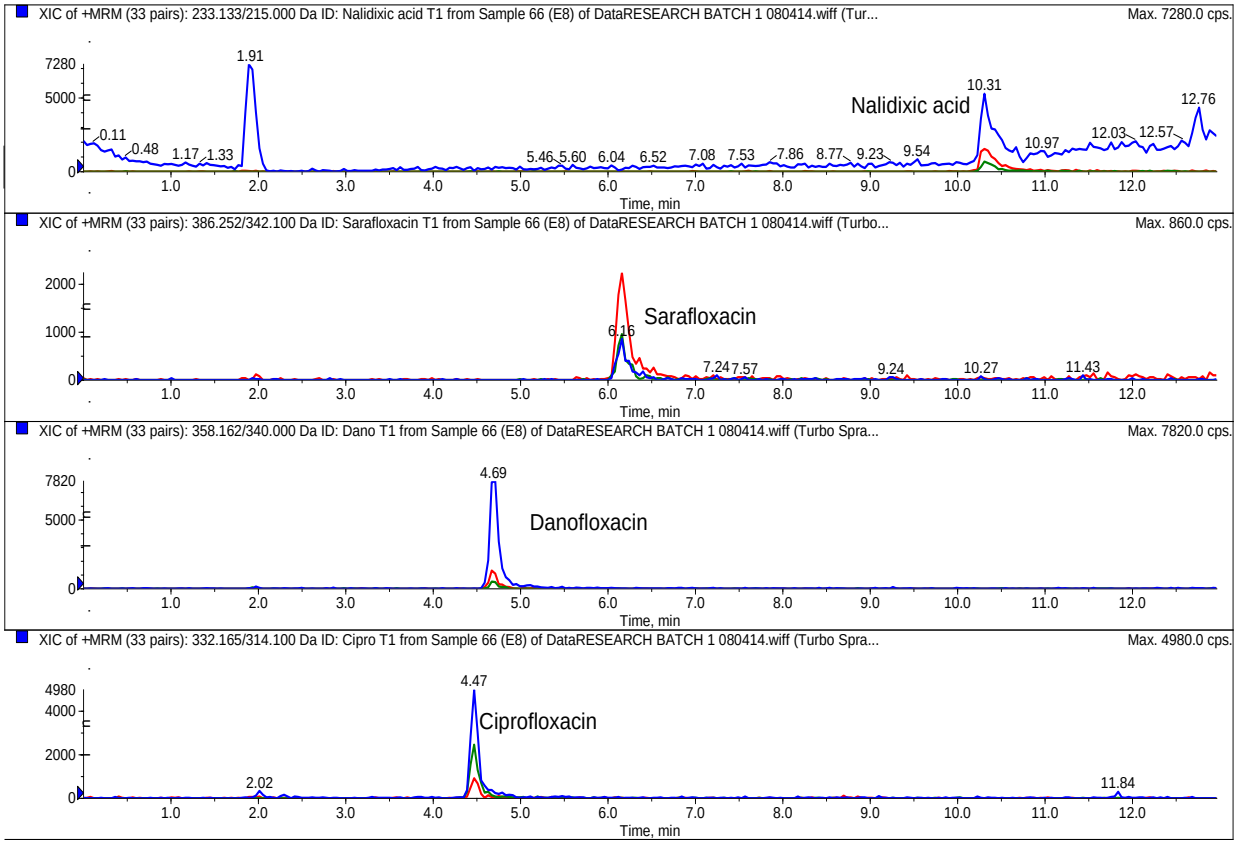


Figure 4.7c: Extracted ion chromatograms of each analyte obtained from the total ion chromatogram in Figure 4.7a; showing retention times of each analyte.

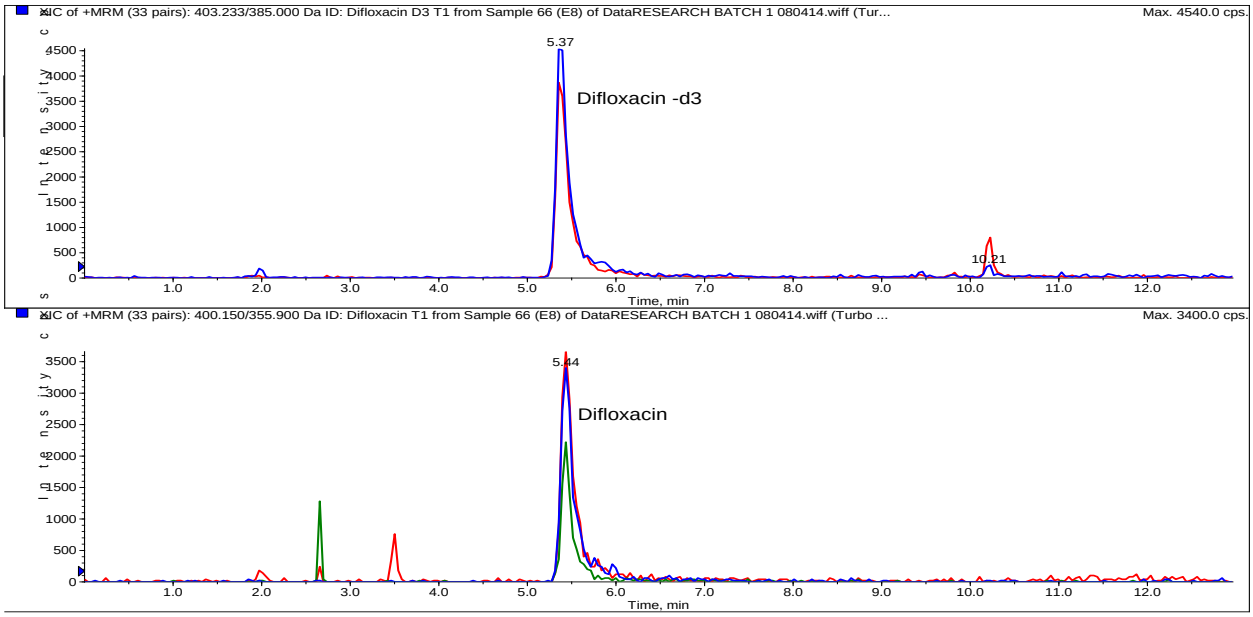


Figure 4.7d: Extracted ion chromatograms of each analyte obtained from the total ion chromatogram in Figure 4.7a; showing retention times of each analyte.

CHAPTER 5: CONCLUSIONS

A greener sample extraction, clean-up and pre-concentration method based on hollow fiber - supported liquid membrane (HF-SLM) was developed as an alternative for extraction of quinolone antibiotics (enrofloxacin, ciprofloxacin, danofloxacin, difloxacin, nalidixic acid, norfloxacin and sarafloxacin) in biological samples such as bovine kidney and analysed by LC-ESI-MS/MS. The method was optimised and validated following the 2002/657/EC directive. The optimized parameters were therefore; 0.1 % formic acid at pH 3 as the acceptor phase, triethylamine as the liquid membrane, NaH_2PO_4 at pH 7 as the donor phase and extraction time of 60 minutes. The validated parameters under optimum conditions were linearity in the range of 0.9714 to 0.9975, LODs (3 to 39 µg kg^{-1}), LOQs (10 to 130 µg kg^{-1}), CCα (28 to 422 µg kg^{-1}), and CCβ (29 to 454 µg kg^{-1}). The method was found to be reproducible with CVs ≤ 23%. When applied to real bovine kidney samples obtained from local abattoirs the results showed that the analysed samples had non-detectable quinolone antibiotics levels based on the extraction method and detection technique used. This implies that withdrawal periods are being complied with before animals are slaughtered. Another sample preparation method employing dispersive solid phase extraction was also developed and validated for extraction of quinolones in bovine kidney. Validated parameters such as linearity (12.5 to 750 µg kg^{-1}), CCα (27 to 429 µg kg^{-1}), CCβ (30 to 464 µg kg^{-1}) were obtained. LOD and LOQs for dSPE also ranged between 3 to 39 µg kg^{-1} and 10 to 130 µg kg^{-1} respectively but the values differ for some analytes. The two sample preparation methods were found to be comparable for some analytes (enrofloxacin, danofloxacin, norfloxacin and nalidixic acid) when statistically subjected to a t-test using recovery data, while there was a significant difference for some of the analytes such as ciprofloxacin, difloxacin and sarafloxacin. From the discussion above, it can be concluded that hollow fiber –supported liquid

membrane can be used as an alternative method for extraction, clean-up and as a pre-concentration method for quinolones in bovine kidney.

5.1 Limitations, challenges and future work

It was discovered during the study when using the hollow fibers that they are very fragile to work with. Possibility of sample loss is very high and there is a need to handle them with care. For future work, it would be a good research area to explore the use of an anion carrier to cater for extraction of acidic analytes like nalidixic acid which is different from other analytes to enhance its extraction. There is also a need to analyse positive samples using HF-SLM to prove that it can be used as an alternative method to d-SPE.

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